Eastwood, Arthur
Bacteriological studies

REPORTS

ON

PUBLIC HEALTH AND MEDICAL SUBJECTS.

No. 13.

BACTERIOLOGICAL STUDIES.

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MINISTRY OF HEALTH.

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To The Right Hon. Sir ALFRED MOND, Bart., M.P.,
Minister of Health.

SIR,

1. I submit four special reports by Dr. Eastwood and his colleagues at the Ministry's Pathological Laboratory on subjects which are of interest and immediate practical importance not only to bacteriologists but to all public health workers.

2. The importance of medical research and particularly of research into pneumonia requires no emphasis. This disease is a substantial cause of national mortality. In 1921 it was primarily responsible for 34,708 deaths in England and Wales apart from those due to diseases in which it is the chief secondary complication, for example, measles, whooping-cough and influenza. Medical Officers of Health and others have long been drawing attention to the need for further investigation in this field to add to the knowledge for which we are indebted mainly to Neufeld and his colleagues in Germany, to the workers of the Rockefeller Institute in America, and to the work carried out by Wright, Lister and others in South Africa.

3. The first three reports deal with the pneumococcus and comprise—

(*a*) A concise and instructive historical survey of the question of serological races among pneumococci by Dr. Eastwood;

(*b*) A study by Dr. Griffith of the strains of pneumococcus found in this country—the most comprehensive investigation of the kind yet undertaken; and

(*c*) A discussion by Dr. Eastwood of the principles underlying the serological differentiation of a bacterial species.

The last is a subject with which Dr. Eastwood is peculiarly competent to deal. It is a criticism of the doctrine of immunity as applicable to the pneumococcus and affects directly the methods at present employed in the preparation of curative sera. It also bears on the question of the significance of the "carrier" (*i.e.*, a healthy person who harbours the germs of infectious disease), a subject to which considerable attention has been devoted in this laboratory, particularly with reference to carriers of meningococci and their relation to the spread of cerebro-spinal fever. The practical application of this work is apparent when we consider the large sums which are being spent annually by local authorities in dealing with carriers of diphtheria bacilli and other germs, and the unnecessary dislocation of social and industrial life which inaccurate or ill-founded conceptions on this subject may entail.

4. In connection with the two first reports, I may recall that at the International Conference on the Standardisation of Sera and Serological Tests, held at this Ministry under your presidency on the 12th–14th December, 1921, it was agreed that international co-operation was needed to ascertain the distribution of different types of pneumococci and to determine the best criteria for the production of effective therapeutic sera. The reports now submitted are a contribution to both problems, and will, it is hoped, help to clear the ground. Publication of carefully considered data about the facts and principles involved shortens the time necessary for the attainment of fuller results, indicates lines of future investigation on which financial outlay is most likely to yield a good return, and guides investigators into channels of enquiry which are not blind alleys but avenues of progressive discovery.

5. The fourth report, by Dr. Scott, on "The Distribution and Serological Characteristics of Influenza Bacilli" embodies the results of an attempt to sub-divide this bacillus, which is very widely distributed, into different varieties or types. It was thought that some types might be found to be less intimately associated with disease than others, and that it might be possible to prove the existence of an epidemic strain. The results, however, have not been encouraging. It formed part of the investigations which were undertaken by our field workers during the recent influenza epidemic and is in continuation of other and similar work incorporated in the report on the epidemic of 1918–1919.

6. The salient features of these reports may be set out briefly as follows :—For many years bacteriologists have been endeavouring to find means of reducing disease and mortality caused by pneumococcal infections, amongst which pneumonia is the most serious. The pioneer work, to which Washbourn and Eyre made valuable contributions, broke the ground and gave rise to hopes that the time would come when serum-therapy would be as successful for the treatment of these diseases as it has proved to be in the case of diphtheria. But there have been many disappointments, owing to the many obscurities and complications of the subject. A fresh stimulus to research was given by Neufeld, who observed that pneumococci were not all of the same serological type, and that special attention must be paid to these differences, because a curative anti-serum would not be effective unless it had been prepared with the same type of pneumococcus as that to which the infection was due. This line of thought has been thoroughly worked out by the investigators at the Rockefeller Institute. They have confirmed Neufeld's view that differences in type impose a sharp limit on the efficacy of an anti-serum; and they have found that the majority of pneumococci isolated from cases of lobar pneumonia are divisible into three main types, though many strains remain

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which are heterogeneous and must, provisionally, be grouped together as "atypical." Further, they have prepared an anti-serum which they have found to be of high therapeutic value in the treatment of pneumonia due to infection with their Type I. These results form a distinguished record of scientific progress. The difficulties which have not yet been overcome are, however, serious. In pneumonia due to Type I, prompt diagnosis of type is needed if the anti-serum is to be used effectively; and special skill is required in the administration of the relatively large doses intravenously. The requisite facilities are not generally available. Again, in the experience of the Rockefeller staff, it has not been possible to prepare a serum which will be useful in the treatment of any case of pneumonia not due to Type I. How are these difficulties to be met? Can they be overcome by further pursuit of the same line of investigation? Or must some new methods be devised? These are the research problems of to-day in this subject.

7. The first task is to level up to the American work. What is the condition, as regards serological type, of the pneumococci responsible for pneumonia in this country? In a series of 150 cases of lobar pneumonia taken mainly from the London area, Dr. F. Griffith finds that the American Types I, II and III occur in about the same proportions as in the United States; he agrees with the Rockefeller workers that these types are serologically distinct, and that there remains a large number of strains which differ from these three types and present many varieties amongst themselves. His own observations have also led him to concur with the opinion, expressed by several of the Americans, that further work is necessary before a final conclusion can be drawn as to the mode of action of anti-pneumococcal serum. In this levelling up process the participation of many investigators is needed, in Great Britain, in the rest of Europe, and in other countries where skilled pathologists are available. As the result of such combined enquiry, and with the support of the Health Committee of the League of Nations, it should be possible to place on record the characters and distribution of the more important types of pneumococci throughout the world. The American Types I, II and III form the starting point; it is for other countries first to ascertain the distribution of these three types and then to add any others which may be found to be of epidemiological importance in the pathogenesis of pneumonia. In this research the results of different workers must be comparable; therefore the technique, which Dr. Griffith discusses with particular care, should be identical in all essential respects. It follows that there must also be uniformity of nomenclature. When this stage has been reached, what next? The ground will then have been cleared for more concerted attack upon the many and difficult problems of pneumococcal immunity which the ingenuity of the Rockefeller staff has not yet been able to solve.

8. In dealing with such obscure questions as these, three methods of enquiry, should be utilised, and to the fullest extent possible, because each depends on the other two for its success. These are—experiment, controversy and reconstruction of knowledge. The need for continued research is obvious if we are to learn the true principles which determine the production of a good therapeutic serum for parasitic bacteria, and how its mode of action is to be explained. About these matters there is much dispute, and it is the proper function of scientific investigation to elucidate them. Immunity is a very young science; it has not yet acquired a solid basis of immutable principles, but has to depend on current theories which are really no more than working hypotheses. It often happens that the immediate result of controversy about a difficult question is merely to emphasise the fact that the controversialists cannot come to an agreement because they are relying on hypotheses which are different and apparently irreconcilable. Thus the qualities of a good serum are explained in a variety of ways, the discussion of which necessarily involves the use of technical terms and leads to questions as to whether the true explanation is to be found in a precipitin, a bacteriotropin, a bacteriolysin, an anti-endotoxin, or an anti-aggressin, and so forth. These theories cannot all be right—so what is to be done with them? If it were possible to take them seriatim and show that, in relation to a concrete problem such as pneumococcal immunity, each of them, except one, is wrong, the matter would be simple. This, however, is not practicable, because most of these theories contain some element of truth and therefore simplification cannot be effected by the method of exclusion. But something may be done by attempting to reconstruct some of these current conceptions about the principles of immunity; and that this should be done is a matter not of choice but of necessity, because these questions are of immediate practical importance to the immunologist and cannot be allowed to remain " held up " indefinitely on the horns of academic dilemmas.

9. The purport of the third report in this volume is accordingly to aid in the adjustment of ideas as to the significance of certain serological reactions. The practical importance of this question in relation to pneumococci is due to the fact that special peculiarities of these organisms appear to involve great difficulties in the preparation of useful therapeutic sera. These difficulties are not likely to be overcome unless further light can be thrown on the nature of those reactions between a micro-organism and its animal host which lead to the production of immunity against parasitic bacteria.

10. Dr. Scott's report provides useful material for comparison between the pneumococcus and Pfeiffer's bacillus of " influenza." Both organisms are very common inhabitants of the upper respiratory passages and are very frequently non-pathogenic,

living as harmless saprophytes on the mucous membranes of normal and apparently quite healthy persons. Both, also, are intimately associated with " colds " and inflammatory conditions though with this difference that the pathogenic rôle of the pneumococcus is accepted, whereas the pathogenicity of Pfeiffer's bacillus as a primary agent of infection is still *sub judice*. It is of particular interest to note that Dr. Scott has found Pfeiffer's bacillus to be frequently associated with the pneumococcus in cases of pneumonia. It is not yet possible to decide whether, in cases such as these, the bacillus prepared the way for the coccus or the coccus for the bacillus; but, in either event, as Pfeiffer's bacillus is known to be toxic, its presence in the tissues cannot be regarded as innocuous. Serologically, both the pneumococcus and Pfeiffer's bacillus form an indefinitely large number of different races; but with the latter organism the differences are not so sharply defined as with the pneumococcus, and there is not the definite association of particular serological types with morbid processes which is found with pneumococci of Types I and II. Like most cautious bacteriologists, Dr. Scott concludes that the plea for Pfeiffer's bacillus as the cause of influenza is neither proved nor disproved. Probably it will take some years before this question is settled. But quite apart from the etiology of influenza, the study of Pfeiffer's bacillus is important because it appears to be one of the organisms which are liable to change suddenly from the saprophytic to the parasitic mode of existence and then to be associated with inflammatory conditions of the respiratory tract and other regions.

I have the honour to be,

Sir,

Your obedient Servant,

GEORGE NEWMAN.

Whitehall,
July, 1922.

BACTERIOLOGICAL STUDIES.

I.—A BRIEF REVIEW OF RECENT BACTERIOLOGICAL WORK ON PNEUMOCOCCI.

By Arthur Eastwood, M.D.

Introduction.

The main problem is to reduce disease and mortality due to pneumococcal infections, amongst which pneumonia is the most important.

For the last twenty-five years or more, the study of the pneumococcus has received wide attention from bacteriologists, but they are still presented with difficulties which have not been solved.

In the earlier work, to which Washbourn and Eyre made important contributions, persistent efforts were made to produce antipneumococcus sera of therapeutic value. These attempts, though not sufficiently successful to become of general utility, have been a helpful stimulus to further enquiry. The next advance was made in Germany by Neufeld and his associates, who laid the foundations for a classification of pneumococci into serological types and endeavoured to show that the recognition of these differences in type must form the basis of work on immunity. Development of this principle is mainly due to the work done in the Rockefeller Institute from 1912 up to the present time, and also to Lister's studies in South Africa on the specific serological reactions of pneumococci and the value of prophylactic inoculation.

So far, progress has been slow. The difficulties which have to be overcome are naturally very great, because so little is known about the biological principles which determine the processes of infection and immunity. More laboratory data are needed.

In this review I have put together what appear to me to be the main items of interest. I have not attempted to make a complete historical record of all the work which has been done in the period under survey, because the work is not all of equal value, and an undiscriminating digest of the whole of it would be more confusing than helpful.

The Starting Point.

For the purpose of considering the present position of the pneumococcus problem, it is convenient to begin with the work of Neufeld and his associates.*

Confining their attention to virulent pneumococci obtained from cases of pneumonia, Neufeld and Händel found that the greater number of these were identical, both serologically and in all other respects. These formed their "typical" group or "Pneum. I." A good serum, prepared by immunising a rabbit, ass or horse with any one of these strains, would protect an experimental animal against infection with a large dose of any strain belonging to this group. But other virulent strains were found which differed from the preceding serologically, though resembling them in other respects. These they termed "atypical." A "typical" immune serum would not protect against an "atypical" strain, nor would a serum produced by an "atypical" strain protect against a "typical" strain. The "atypical" strains, again, were subdivided into serological groups; a serum prepared with a member of one group would protect against each of the members of that group but not against any members of another group. They had already, in 1912, found three such groups and expected that extended investigation would reveal more.

In protection tests on animals they found that the serum did not act in accordance with the law of multiple proportions. For example, 0·2 c.c. of a serum protected mice with certainty against 0·1 c.c. of culture, which was at least 100,000 times the lethal dose; but one-tenth of this dose of serum gave no protection against 0·01 c.c. of culture and failed to give regular protection against much smaller doses of culture; and, when the quantity of serum was reduced from 0·2 to 0·002 c.c., its protective power had entirely disappeared. From many observations, made by them and other investigators, they came to the general conclusion that the quantity of serum injected must attain a certain

* The article on pneumococci by Neufeld and Händel in Kolle and Wassermann's *Handbuch der pathogenen Mikroorganismen* (2nd Edition, Vol. IV., 1912) gives a good summary of their own work and its relations to that of other investigators.

"threshold value," *i.e.*, a certain concentration in proportion to the body weight of the animal, before it exercised its protective action. When this quantity of serum was introduced, protection was valid against a very large multiple of the minimal fatal dose.

From their animal experiments, they thought it possible that cases of pneumonia in man might be successfully treated with antipneumococcus sera, provided that three conditions were fulfilled. (1) The serum must be of high titre in protection tests on mice; (2) it must be prepared with a pneumococcus strain of the same serological type as the strain infecting the patient; and (3) it must be injected intravenously in large doses, *e.g.*, 75 c.c., in order to attain the requisite concentration in the patient's body. They considered that it would be wise to concentrate first on the serological treatment of infections due to their "typical" group of pneumococci, and to wait until the value of this method was established before attempting similar work on infections where the organism was one of the "atypical" varieties.

They offered the above suggestions as a starting point for future enquiries and made it clear that they were not, at the time of writing (1912), in a position to express any judgment upon the value of serum therapy in the treatment of pneumonia.

Progress.

American Types.

This starting point would have led to very encouraging results if, on more extensive enquiry into cases of pneumonia, Neufeld and Händel's tentative and cautiously worded suggestions had been substantiated in three respects. It might have been found (1) that "Pneum. I" was responsible for the majority of cases of pneumococcal pneumonia, (2) that, in the remaining cases, the pneumococci present were divisible into a reasonably small number of serological groups, and (3) that, for each of these groups, as for "Pneum. I," specific sera could be produced which were effective not only in protection tests on mice but also in the treatment of human pneumonia. Unfortunately, these anticipations have only been realised to a limited extent.

American investigators have found that the most important group of strains in their country is Type I, corresponding to Neufeld's "typical strains." In 1917 the Rockefeller Institute reported that this type occurred in 33·3 per cent. of a series of 454 cases of lobar pneumonia. The serological characters of this type are sharply defined; it is definitely associated with disease and is very rarely found in normal individuals, except in contacts with cases of pneumonia. The next American group is "Type II (typical)." This is serologically distinct from I, but resembles it in its definite, and almost exclusive, association with disease. It accounted for 29·3 per cent. of the above series of lobar pneumonias. Then there come a large number

of groups which the Americans term, collectively, "Type II "(atypical)." These have been investigated by Stillman.* They are related to Type II, because they are agglutinated by concentrated Type II serum, though not by the diluted serum, *i.e.*, they "possess partial antigenic characters common to the "Type II pneumococcus." They are distinguished from typical II "by a diversity of relations among themselves, and by a "lack of the reversibility of their immune reactions with the type "organism." Stillman examined 204 "atypical II" strains and classified them into 12 distinct groups, on the basis of specific agglutination in monovalent rabbit sera. He reported that, out of 458 strains obtained from lobar pneumonias at the Rockefeller Institute in three years, 52 (11 per cent.) were "atypical II." These "atypical" groups are not limited to cases of disease. According to Stillman, they occur in 18·3 per cent. of normal mouths. The next type, Type III or *Pneumococcus mucosus*, possesses distinctive characters and is of common occurrence in normal mouths. It accounted for 13 per cent. of the Rockefeller series of lobar pneumonias published in 1917.

So far, then, the Americans have made out fifteen serological groups of pneumococci. There remain a large number of strains which they have not been able to place in any comprehensive system of groups, owing to the great diversity of their serological reactions; they have shown, however, that they do not belong to any of the above 15 groups. This heterogeneous collection they term "Type IV." This "Type" is very common in the normal mouth. It accounted for 20·3 per cent. of the last mentioned Rockefeller series of pneumonias.

With reference to the relation of type to virulence, the same Rockefeller report gives the following figures, which refer to 100 cases of lobar pneumonia not treated with serum.

Type of pneumococcus.	Incidence per cent.	Mortality per cent.
I.	33	25
II.	31	32
III.	12	45
IV.	24	16

As regards serum therapy, they have produced a horse serum which is useful in the treatment of pneumonia due to infection with Type I, but they have not succeeded in obtaining a serum which could be used for infections due to Types II or III or the miscellaneous "Type IV." Evidence as to the value of Type I serum has recently been given by Cole.† In 195 cases treated with this serum at the Rockefeller Institute there were only 18 deaths (9·2 per cent.). He has collected from the literature records of 300 additional cases treated with the serum and finds

* *Journ. Exper. Med.*, XXIX, p. 251, 1919.

† *Journ. Am. Med. Ass.*, Jan. 8th, 1921, p. 111.

that the mortality for the total 495 cases is 10·5 per cent.* The serum is given intravenously in doses of 90–100 c.c., repeated every eight hours until there is a satisfactory fall of temperature and improvement of symptoms. Cole states that the average amount of serum required per case is from 200 to 300 c.c., but in severe cases, treated late in the disease, larger amounts are needed, even up to 1,000 c.c.

South African Types.

In South Africa, Lister has attempted a serological classification of strains of pneumococci obtained from cases of pneumonia amongst native miners. During the period 1913–17† he collected 148 strains and found that these were divisible into 12 groups. His Group C (American I) contained 32 cases (21·6 per cent.); Group B (American II) contained 24 (16·2 per cent.); and Group E (American III) contained only 2 cases. The rest, apparently, would all be relegated by the Americans to the "scrap-heap," Type IV, including Lister's Group A, which was the most important of the twelve, as it contained 46 cases (31 per cent.).

Lister vaccinated miners with cultures of his three most important groups and found that protection was conferred against pneumonia attributable to members of these groups.

Multiplicity of Agglutinins.

From the above brief outline it would appear that antigenic differences amongst pneumococci are extremely numerous, that the search for such differences is far from being completed, and that there is no definite prospect of arriving at a stage of finality.

It should be mentioned, however, that some investigators have found evidence of serological relationship amongst strains more usually regarded as antigenically distinct. Miriam Olmstead,‡ for example, found that members of "Type IV" were not quite so heterogeneous amongst themselves as was originally supposed. By means of agglutinating rabbit sera, she succeeded in classifying 94 "Type IV" strains into 12 serological groups. Mildred Clough has stated§ that, amongst the pneumococci isolated from pneumonias and other cases in the Johns Hopkins Hospital during 1915–18, nine strains agglutinated with sera of all three types (I, II, and III) in fairly high dilution (1 : 8 to 1 : 64 or higher), while normal horse serum caused no agglutination. In confirmation of these results, she found that the three type sera also stimulated active phagocytosis of all the strains, though there was no phagocytosis with normal horse serum controls; and the nine strains elaborated in the bodies of

* Cecil and Blake have also used this serum successfully on monkeys experimentally infected with doses of Type I pneumococci which were fatal to the controls (*Journ. Exper. Med.*, XXXII p. 1. 1920).

† *Publications of the South African Institute for Medical Research* No. 10. November, 1917.

‡ *Journ. Immunol.*, II, p. 425. 1917.

§ *Journ. Exper. Med.*, XXX, p. 123. 1919

inoculated mice a soluble substance which gave a precipitate when the peritoneal washings, cleared by centrifugalisation, were added to each of the three type sera. Absorption of I and II sera with I and II strains removed the agglutinins and bacteriotropins for all her nine strains, but absorption with one of these nine strains removed these substances for itself only, not for I or II strains nor for the other 8 strains. She prepared immune sera with her strains and found that they were serologically distinct, though with some interrelationship between certain of them; the sera had no activity towards strains classed as Type I or Type II or "atypical II." The suggestion of underlying interrelationship between antigenically different strains is also provided by the work of Laura Stryker,* who observed the effect of prolonged culture in homologous serum. After this treatment, Type I strains to a large extent lost their agglutinability with Type I serum, and became agglutinable with Type II serum; Type II strains gave corresponding results, losing their agglutinability by the homologous serum and becoming agglutinable by Type I serum. These changes in serological reactions were accompanied by loss of virulence and reduction of antigenic capacity.

It will, of course, be recognised that instances such as those which I have quoted do not carry much weight and can only be regarded as minor exceptions from the general rule. The main fact which remains is that antigenic differences amongst pneumococci are closely reflected in the sharp differences between the agglutinins produced by different strains.

Precipitin Tests.

The precipitin test, whilst affording indications that all strains of pneumococci possess something in common, gives results which correspond very closely with those obtained by agglutination. Blake,† who has paid special attention to differentiation of pneumococci by this test, gives the following example :—

Dilutions of Type II antigen (0·5 c.c.)	1·100	1·500	1·1000	1·5000	1·10,000	1·50,000
0·5 c.c. of Serum I (1·10)	++	±+	+	±	−	−
,, ,, II ,,	+++	+++	+++	+++	+++	++
,, ,, III ,,	++	++	±+	+	±	
,, ,, (Normal) ,,	−					

The reactions were read after two hours at 37° C. and overnight on ice. The antigen was prepared by drying *in vacuo* the washed pneumococci from an 18-hour 1,000 c.c. broth culture; the dried material was taken up in saline (10 mg. per c.c.) and repeatedly frozen and thawed until a faintly opalescent fluid free from bacterial bodies was obtained.

* *Journ. Exper. Med.*, XXIV, p. 49. 1916.
† *Journ. Exper. Med.*, XXVI, p. 67. 1917.

He states that "similar results have been obtained with "antigen prepared from strains of Pneumococcus Type I and "Pneumococcus Type III. Group IV pneumococcus antigens "show a precipitin reaction only within the limits of the "non-specific zone."

Specific Soluble Substances.

Dochez and Avery* have shown by means of the precipitin test that pneumococci elaborate a specific soluble substance during the first 12 hours of their cultural growth, *i.e.*, at a time when little or no death has occurred in the cultures. "This "would seem to indicate that this soluble substance is not the "result of bacterial disintegration, but represents an actual "extrusion of the cell substance into the medium during the life "processes of the organism."

They also found that similar substances were developed in the animal body. For example, they inoculated a rabbit intraperitoneally with 1 c.c. of the blood of a rabbit infected with Type II, took blood from the heart at frequent intervals, and tested the bacteria-free serum. Against the homologous immune serum their results were :—before infection —, after 2 hours $\pm$, after 4 hours + $\pm$, after 6 hours + +, after 8 hours + +. Control tests with Type I serum were all negative. They also found that Type II serum gave a marked precipitate with the rabbit's urine taken at the end of 24 hours. In human infections, they found that lobar pneumonia patients "have in "their blood and more frequently in their urine a specific soluble "substance of pneumococcus origin." The presence of a large amount in the urine was to be regarded as an unfavourable sign. Their general experience was that Type III strains formed "specific soluble substance" more abundantly than Type II, and that Type II formed more than Type I. These phenomena might be associated with the property of capsule formation, which was most developed with Type III, the capsules of Type II being smaller and those of Type I still smaller. Their specific soluble substance was not destroyed by boiling; it was precipitable in acetone, alcohol, and ether, and easily redissolved in water. It did not dialyse through parchment and its immunological reactions were not affected by digestion with trypsin. They considered it "to be of protein nature or to be associated with "protein."

In connection with this subject, Cole's work† may be mentioned on the neutralisation of antipneumococcus immune bodies by infected exudates and sera. He found that in some cases, though not invariably, fluid from the thoracic cavity in cases of empyema following pneumonia reduced or abolished the agglutinins and protective substances of the immune serum

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* *Journ. Exper. Med.*, XXVI, p. 477. 1917.
† *Journ. Exper. Med.*, XXVI, p. 453. 1917.

homologous to the coccus which had caused the infection. In infected rabbits which had developed septicæmia, inhibitory substances appeared in the blood; and, when immune serum was injected into infected rabbits, the immune substances disappeared very quickly. "When immune serum is administered to patients "severely infected with pneumococci, the immune bodies may also "disappear very rapidly, and this disappearance is probably "associated with the presence of such soluble substances in the "blood." With reference to the observations of Dochez and Avery on specific precipitable substances, he says :—"While it is not "certain that the substances in the infected animals which give "rise to fixation of antibodies are identical with those concerned "in the precipitation phenomenon, it seems likely that this is the "case." The reason why Type II serum was less effective than Type I might be "not only because its concentration of immune "bodies is less than that of Type I serum, but also because the "power of pneumococci of this type to produce fixing substances "is more highly developed than is that of pneumococci of Type "I." Apparently a similar reason might account for the relative inefficacy of Type III serum.

Protection Tests on Mice.

The results of protection tests on mice are often found to run parallel with those obtained by agglutination and precipitation, *i.e.*, the potency of the immune sera may appear to be equally well demonstrated by each of the three methods. But this parallelism is not found invariably. The Rockefeller investigators have discussed this question in relation to the standardisation of immune sera and have advanced reasons for adopting the protection test on mice as the best criterion. They say* :—"In any case, however, the hope of devising a method of "standardisation, employing either agglutination or bacteriotropic "action, has been destroyed by the observation that, while there is "frequently some gross quantitative relationship between the "protective action of a serum and its agglutinating or bacterio-"tropic power, this relationship is inconstant. For instance, we "have had sera with high protective power and little or no "agglutinating power and *vice versa*."

Experiments on Monkeys and Rabbits.

Recent experiments on monkeys indicate that the problem of pneumococcal immunity in relation to serological types is somewhat complicated.

Cecil and Blake† vaccinated monkeys by subcutaneous injection of living cultures of Type I and found that active

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* *Monographs of the Rockefeller Institute for Medical Research.* No. 7 p. 54. 1917.

† *Journ. Exper. Med.* XXXI, p. 657 and p. 685. 1920.

immunity was produced not only against Type I but also, though in variable degree, against other types of pneumococci. This, they say, " confirms the fact already established that the various " types of pneumococci are closely related biologically." They also state that monkeys, which had survived experimental infection with Type I and were immune to subsequent infectio with this type, sometimes, though not invariably, showed " a " certain amount of cross-immunity against the other fixed types." But these results did not apply to Type IV. Experimental pneumonia due to this type " confers slight if any protection " against subsequent infection with the same or with an homolo- " gous strain of Pneumococcus Type IV." And Type IV pneumonia did not confer any immunity against Type I nor, conversely, did Type I pneumonia immunise against Type IV. In their monkeys which had been immunised, either by vaccination or by recovery from experimental pneumonia, there was no constant relationship between active immunity and the presence of agglutinins or protective substances in the animal's serum. " The serum of a " monkey may be entirely free from these substances, and yet the " animal may possess a high grade of immunity against pneumonia. " On the other hand, . . . the serum of a vaccinated monkey " might protect mice against 100 or even 1,000 minimal lethal " doses of pneumococci, and still that monkey be susceptible to " experimental pneumococcus pneumonia. These facts complicate " the whole question of resistance to pneumococcus infection and " revive the old problem of humoral *versus* cellular immunity."

To these observations may be added a short note on the later work of Cecil and Steffen.* They found that the subcutaneous inoculation of monkeys with three large doses of Type I vaccine conferred complete immunity against experimental Type I pneumonia, and that the same results were obtained with small doses of the vaccine when used intravenously. But the appearance of protective bodies for mice in the serum of their immune monkeys was quite irregular and might not occur. " There " appears," they say, " to be no intimate relation between active " immunity against pneumonia and the presence or absence of " protective substances in the serum of the vaccinated animal."

The above observations, which suggest that serological reactions do not furnish a complete explanation of the mechanism of immunity, correspond in some respects, though not in every detail, with the earlier results of Cole and Moore. These investigators† found that animals whose sera were protective and curative were always highly immune; but " an animal may " itself be fairly highly immune without the serum containing any " immune bodies that we can demonstrate, and without its having " any demonstrable protective action." For example, they immunised rabbits to withstand 0·1 c.c. of a culture, of which

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* *Journ. Exper. Med.*, XXXIV, p. 245. 1921
† *Journ. Exper. Med.*, XXVI, p. 537. 1917.

0·000 001 c.c. killed the control; but the serum of these animals had no protective power and showed no trace of immune bodies. Possibly, they thought, there might be a fundamental difference between active and passive pneumoccocus immunity; or, at any rate, in resistance and recovery "there may be other factors "concerned than the humoral ones." They considered that the action of immune serum did not depend entirely either on its agglutinative or on its bacteriotropic power. "The protection "of small animals undoubtedly reproduces more accurately the "part which the serum plays in recovery from natural infection, "as seen in the human patient, but even here the conditions are "not identical . . . The production of active immunity is "attended with little difficulty and the form of antigen, so far as "we know, is not of great importance, but when we come to the "question of the production of humoral immunity, especially of "the highest grade, this factor may be of the greatest importance." Virulence for the mouse and rabbit did not give much clue to the virulence of a strain for man. It was not known whether it was important or not for the cultures to be of high virulence when used for producing immune sera. They observed that Neufeld and his colleagues said that it was, but did not give protocols to prove it.

Inhibitory Properties of Immune Serum.

Dochez and Avery* raised the question whether the action of pneumococcal immune serum might in some respects be comparable to Ascoli's "antiblastic immunity" in anthrax. They thought they were able to show that their immune sera did exercise some inhibitory influence on the growth of cultures. For example :—

Serum 0·1 c.c.	Culture 0·000 01 c.c.	No. of colonies.	
		Immed.	After 3 hours.
Antipn. Serum I -	Pn. I	230	216
„ „ II -	„	200	630
Normal „ -	„	200	1,400

But this retarding influence only lasted for a short time; when plates were made after 24 hours, it was found to have been completely overcome.

They considered that agglutination and thread formation could not be entirely responsible for the temporary inhibition of growth, because "a marked delay in development occurs in "heterologous immune serum in which no agglutination whatever "takes place and in which thread formation is not more extensive "than in normal serum." Their view was that there had been

* *Journ. Exper. Med.*, XXIII, p. 61. 1916.

" active interference with the growth phenomena of the organisms." They also found that immune serum reduced the output of amino nitrogen in cultures after 24 hours incubation and that it interfered with the capacity of the coccus to ferment inulin. They suggested that integrity of the digestive zone at the surface of the bacterial cell was necessary for growth, that the action of anti-enzymotic bodies was to interfere with this zone, and that capsule formation was possibly an attempt to protect the function of the digestive zone.

Blake,* however, whilst admitting that specific serum apparently caused some inhibition of the metabolic activities of pneumococci, thought that this was merely due to agglutination and that there was no evidence of a specific antienzymotic property of the serum. When exhausted of its agglutinin content, the serum possessed no inhibitory properties, and these properties were found to be in direct proportion to agglutinating power. In estimating growth by plating methods, it was obvious that. when the cocci grew in clumps and chains, a sample would give fewer colonies than the controls. Agglutination made the cocci unable to grow in intimate contact with the whole medium; but, when the cultures were frequently shaken, the metabolic activities were the same in homologous serum as in the controls.

Barber† applied his " single cell method " to the study of the problem; he picked out, under the microscope, single pairs of pneumococci and observed what happened to them when they were introduced into fluids which were supposed to be " antiblastic." He found that growth of a single pair in immune horse serum proceeded as fast as in the controls, though the character of the growth was distinctly different. " The cells " early became invested with a thick capsule and grew in chains " which often intertwined and formed zooglea-like masses . . . " The capsule formed also on cells which were isolated from old " cultures and were apparently dead or, at all events, subsequently " showed no growth in hanging drops." He also made cultures in test-tubes which were inoculated with one or two pairs and subsequently counted to determine the number of generations which had formed in a given time; he found that there was practically no difference between growth in homologous and heterologous serum, in spite of the formation of clumps in the former. These results were not altered when complement was added to the homologous serum.

After taking a sample of blood from a rabbit, he inoculated the animal intravenously with 5 c.c. of Type I serum and took a second sample of blood an hour later. The rate of growth in blood, and also in serum, was the same in the two samples. Again, experiments with the whole blood, serum and plasma of the rabbit three days after receiving the immune serum showed

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* *Journ. Exper. Med.*, XXVI, p. 563. 1917.
† *Journ. Exper. Med.*, XXX, p. 569 and p. 589. 1919.

that the immune serum had no measurable influence on the rate of growth.

Cocci from the peritoneal cavity of mice, which had been immunised with 0·2 c.c. of serum one hour before the injection of culture, grew in hanging drops at the normal rate.

And in the peritoneal cavity of the immunised mouse the rate of growth appeared to be normal. A mouse was given 0·2 c.c. of serum and was inoculated with culture 1½ hours afterwards. The culture had been slowly centrifugalised for 5 minutes and only the upper part (free from chains) was used. The rate of growth was calculated by counting the number of pairs of cocci per chain after 2, 60, 90 and 120 minutes. Apparently growth continued until the chains or groups were overtaken by phagocytosis, and "the growth of some portion, at least, of the cells "continues at a geometric rate." He observed that "capsule "formation was marked in the peritoneal cavity of the immunised "mouse, but in Type I pneumococcus infection this apparently "interfered little with phagocytosis."

Barber considered that the mechanism of passive immunity was obscure. He did not think that agglutination was "of "paramount significance" and he considered that "the exact "importance of the property of facilitating phagocytosis has also "not been thoroughly determined."

He also made observations on the blood in acquired and in natural immunity. The whole blood (diluted 1 : 11) and the serum (diluted 1 : 5) of an immunised horse were tested in hanging drops and in test-tubes. There was no difference in the rate of growth between the immune blood and serum tests and the controls. Similar results were obtained with whole, undiluted, citrated blood. Hence he found no evidence that "inhibition of "growth of pneumococci by serum or body fluids plays any part "in acquired immunity." He compared, by the hanging drop method, fresh, citrated, undiluted pigeon blood (1), with rabbit blood (2). The growth in (1) was about one generation behind (2); chains were formed in (1) and discrete elements in (2). As the result of further experiments, he found that "growth occurred "about as often in the pigeon as in the rabbit blood, and the rate "of growth in both was practically the same."

In the preceding paragraphs I have confined myself to a statement of American evidence for and against the application of an "antiblastic" theory to pneumococcal immunity. An account of Ascoli's work and a discussion of its significance are contemplated as part of the subject of a subsequent report from me.

Interrelationship of Types.

From what has been said in the preceding pages, it will be noted that slight evidence of interrelationship may sometimes be obtained by agglutination or precipitation tests or by immunisation experiments on monkeys. The facts, however, which stand out much more prominently, as the combined results of experiments *in vitro* and *in vivo*, are (1) the sharp differences

between one "Type" and another and (2) the extremely wide range of antigenic differences to be found amongst the "atypical" strains.

Leucocytes as an Adjuvant for Immunisation.

Is it possible to improve the value of a serum by employing some adjuvant along with the pneumococci used for immunisation?

Some slight support to this suggestion is afforded by the work of Alexander.* Observing that, in human pneumonia, serum taken about the time of the crisis often has some protective power, and that therefore it is possible for the living body to elaborate protective substances within a short time, he tried to find a rapid method of producing immunity in rabbits. The adjuvant which he tried was rabbits' leucocytes. He worked with Type II pneumococci and Type II immune horse serum, both obtained from the Rockefeller Institute. His cultures were washed in saline and sensitised by adding excess of immune serum, precipitation being apparently complete in about 4 hours. The serum was removed by centrifugalisation and the cocci were then emulsified in saline. The cocci and an emulsion of leucocytes were mixed together and incubated in a water-bath at 37° C. for 6 to 8 hours.

Unsensitised cocci, after incubation with leucocytes, and also cocci which were sensitised but not incubated with leucocytes, were too virulent to use in large doses. Cultures treated by his method were attenuated but did not lose their vitality. The cocci and leucocytes ought not to be incubated for more than 6–8 hours. When he incubated them for 11½ hours the rabbits became ill and died.

The injections were made intravenously. A rabbit received 3 injections, on successive days, of 1,000 million cocci and 100 million leucocytes. Blood was taken 5 days after the last injection and it was found that 0·2 c.c. of the serum protected mice against 0·001 c.c. of culture, 0·000 000 01 c.c. being fatal to the control. He tried to increase the amount of protective substance by giving rabbits three series of injections, but did not succeed. He also tried the effect of treating his sensitised cocci with leucocytic extract instead of whole leucocytes but the results were less uniformly successful. When both leucocytes and sensitised cocci were thoroughly washed to get rid of all traces of serum, the results were irregular, apparently because traces of free serum assisted the action of the leucocytes.

The Work of Preston Kyes.

An entirely new departure in serum therapy has been made by Preston Kyes, who has endeavoured to cure human pneumonia by treatment with serum obtained by inoculating fowls. It is difficult to explain his results, or to appraise their exact value; his work is worth quoting because it is just possible that his

* *Journ. Med. Res.*, XXXVII, p. 471. 1918.

serum may have the property of stimulating the production of protective (? bactericidal) substances in the living body.

He has been producing this serum since 1911 and in a recent article* he gives the following data as to its efficacy. In the winter of 1916–17, 653 cases of acute lobar pneumonia were admitted to a hospital in Chicago. They were admitted in rotation, and without discrimination, to eight wards. In seven wards (538 cases) no serum treatment was given; the eighth ward (115 cases) received this treatment. In this last ward the death rate was 20·8 per cent.; in the other wards it was 45·3 per cent. The serum was given intravenously in average doses of 2·5 c.c. Many patients received only one injection per day; others had two per day. The injections were continued until the temperature remained below 100° F. The number of injections per case ranged from 1 to 12, the average being 3.

Kyes' serum has also been tried by Major A. W. Gray at the Base Hospital, Camp Grant, Ill.† Between 1st October, 1917, and 20th September, 1918, 322 cases of typical lobar pneumonia were treated. The death rate was 7·7 per cent. There were no controls, but it was evident to the clinicians that the serum was of marked value. Further details of the above series of cases are given by McClellan,‡ who tabulates the results as follows :

	Type.				Not Typed.	Total Series.
	I	II	III	IV		
Cases - - - -	43	56	13	199	11	322
Deaths - - -	4	8	0	13	0	25
Percentage mortality -	9·3	14·2	0	6·5	0	7·7

Between 21st September and 4th November, 1918, 234 cases of " epidemic pneumococcus—broncho-pneumonia " were treated, with a death rate of 16·7 per cent.; in 1,684 control cases the death rate was 53·6 per cent. From 5th November, 1918, to 1st May, 1919, serum treatment was given to 118 cases, with a death rate of 4·3 per cent.; " these were atypical cases of " pneumococcus pneumonia, which seemed to indicate a gradual " change from the epidemic type of disease back to the type of " lobar pneumonia, probably due to a falling virulence in the " organism." The serum was always given intravenously. " Shortly before the epidemic the dose was increased from 2·5 c.c. " once or twice daily to 5 or 10 c.c. twice daily, and this larger " dosage was maintained during the epidemic. In a few cases as " much as 30 c.c. was given daily. Commonly a total of 60 to " 90 c.c. was given in cases that recovered."

* *Journ. Med. Res.*, XXXVIII, p. 495. 1918.
† *Am. Journ. of Med. Sciences*, CLIX, p. 885. 1920.
‡ *Journ. Am. Med. Ass.*, LXXII, p. 1884. 1919.

In the preparation of his serum, Kyes obtained cultures from the blood or lungs of patients with acute lobar pneumonia. They were grown for 48 hours on blood-agar, slanted in flasks which gave a surface equal to 20 ordinary test-tube slants, and were inoculated (alive) intraperitoneally. The initial dose was usually equal to about 240 test-tube slants, and the average subsequent doses equalled about 400 slants. Injections were made every other week and the total period of inoculation varied from four months to 2 years. Serum was obtained by bleeding the fowls once a fortnight, the week of bleeding alternating with that of injection. "In a few groups bleedings were commenced "following the 6th injection, but the bulk of the serum employed "in the clinical tests was taken from the fowls having received "not fewer than eight nor more than thirty inoculations. The "blood was drawn by incising the leg vein and from 20 to 40 c.c. "obtained from each fowl at a bleeding. The blood corpuscles "were removed by centrifugalisation and the serum, diluted one-"half with ·85 per cent. NaCl, was filtered through Berkefeld "candles." Kyes found that the sera possessed a high content of specific antibodies, but he did not regard content of agglutinins as an index of the therapeutic value of a serum. A univalent high titre serum (1 : 100,000) was tested against 77 strains of all sorts and agglutinated them all (nine only up to 1 : 20, but the majority up to 1 : 500). He thought it open to question whether weak sera really established fundamental differences between different strains. "On the other hand, it is of course well "established, and quite apart from any particular classification, "that various strains of pneumococci do display distinct differences "at a given time and also that a given strain displays quite as "distinct differences at various times. In producing each lot "of serum, therefore, I have inoculated a great variety of strains, "including four of *Pneumococcus mucosus*."

Recent French Investigations.

A brief account must now be given of French work on the serological classification of pneumococci and the production of immune sera.

In 1918, Nicolle, Jouan, and Debains* expressed the view that the classification of pneumococci was greatly simplified by adopting the method of Porges, who had found that capsulated bacilli could be made agglutinable by treatment with dilute acid and subsequent neutralisation. After applying this treatment, they found that all pneumococci became agglutinable by the serum appropriate to their race, and that the American scrap-heap ("Type IV") disappeared. Their technique was as follows. The centrifugalised deposit of a liquid culture was suspended in saline (1 centigramme to 20 c.c.). To this was added 0·1 c.c. of normal HCl; sometimes more acid was necessary (from 0·2 to 0·5 c.c.). Then the tube was immersed for five

* *C. R. Soc. Biol.*, Vol. 81, p. 839.

minutes in boiling water, cooled under running water, and neutralised with normal NaOH.

In 1919, Nicolle and Debains* discussed the principles on which their classification was based and gave their results in detail. Their theory was an amplification of the "mosaic pattern" conception of antigens. Every bacterium contained an unknown number of certain constituents, termed antigens, which were capable of producing agglutinins and lysins in the animal body. The apparent isolation of an antigen merely meant that one antigen was found to predominate and to obscure the other antigens which were also present and might be demonstrated by other methods. Thus, agglutination emphasised a particular antigen and so formed the criterion for racial characteristics, whereas the "fixation" test revealed the existence of all the antigens characteristic of the species, and so formed the criterion for the determination of species. Antigens were the units ("corps simples") of immunology, not the exclusive properties of particular cells. Thus there was nothing surprising to find that certain antigens were common to pneumococci, streptococci and cholera vibrios; and it was wrong to explain the serological demonstration of this fact as due to "heterologous" action.

They tested the agglutinability of a large number of strains of pneumococci (all bile-soluble) and found that 7 per cent. were hyperagglutinable (*i.e.*, agglutinated in normal serum), 48 per cent. were agglutinable without treatment, and 45 per cent. were agglutinable after treatment by their modified Porges' method. For the preparation of their sera they selected four standard strains, designated I, II, II, IV. The first three corresponded with the American I, II and III. Their IV was a special type, isolated from negroes and therefore not to be confused with the heterogeneous American "Type IV." With these strains Truche immunised horses and obtained sera each of which agglutinated only its homologous strain. Their classification was as follows:—

Agglutinable Pneumococci.—*Pure types*: 70 per cent., *viz.*:—I, 1 per cent.; II, 32 per cent.; III, 59 per cent.; IV, 8 per cent. *Mixed types*: 30 per cent. *viz.*:—I + II (I dominant), 64 per cent.; "without dominance," 9 per cent.; II + III (II dominant) 9 per cent.; IV + II (IV dominant), 9 per cent.; I + II + IV (I and II dominant), 9 per cent.

Pneumococci Inagglutinable before Treatment.—*Pure types*: 91 per cent., *viz.*:—I, 9 per cent.; II, 82 per cent.; III, 3 per cent.; IV, 6 per cent. *Mixed types*: 9 per cent., *viz.*:—II + I (II dominant), 33·3 per cent.; II + IV (II dominant), 33·3 per cent.; II + IV ("without dominance"), 33·3 per cent.

Hyperagglutinable Pneumococci.—II dominant, 60 per cent.; II + IV dominant, 40 per cent.

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* *Bull. de l'Acad. de Méd.*, p. 843.

They remarked that the frequent emergence of II antigen was not to be attributed to a greater strength of their II serum.

The following is the method employed by Truche for the preparation of his sera.* The deposit from cultures 15–18 hours old is treated with alcohol-ether, dried in *vacuo*, and rubbed into a very fine powder. His horses receive, intravenously, 2 centigrammes on 10 successive days; after 10 days rest, the horse is bled. A fortnight later, gradually increasing doses are given on 4 four successive days and are followed by 6 days' rest; on the 11th day the horse is bled. The process of immunisation is then continued as before. He tests his sera by agglutination, fixation of complement, and protection. For the last test he inoculates 0·1 c.c. of serum subcutaneously into a mouse and the next day gives a subcutaneous dose of 0·01 c.c. of the homologous culture, the virulence of which must be such that the control is killed by a millionth of a c.c. or less. He does not find that the preventive or curative powers of his sera run parallel with their agglutinating capacities. Following the "mosaic pattern" conception, he considers that the organism responds to the accessory antigens and not only to the dominant antigen. "The serum obtained therefore offers a zone of protection which "is certainly wider than that of the dominant antibody. Notably "with man, a serum possessing a strong preventive power is active "against the homologous type and also against certain heterologous "types; hence it is that a certain number of cases of pneumonia "of Type III have been cured by serum I and, more particularly, "by serum II."

Several French clinicians have made favourable observations as to the efficacy of Truche's sera.

Difficulties.

Whilst appreciating the value of the progress which has been made, it must be admitted that the present position is unsatisfactory. The Rockefeller investigators can only offer serum therapy if the case is found to be due to Type I; in that event, their experience is that large and repeated intravenous injections of specific serum will bring down the mortality due to this type from about 25 to about 10 per cent. Diagnosis of the type should be made at an early stage of the disease and treatment should follow immediately. But, in hospitals, patients are often in an advanced stage on arrival; and, owing to the special skill and care which are required and to the very large quantities of serum needed, this treatment is not likely to be readily adopted by the general practitioner.

If the precise antigenic characters of the infecting strain of pneumococci are all important, one can understand that it would be difficult, if not impossible, to provide therapeutic sera which would be useful for infections with "atypical" strains; but, even on this assumption, there is no generally accepted explana-

* *Ann. de l'Inst. Pasteur*, XXXIV, p. 98. 1920.

tion why immunisation of horses with the second and third of the "fixed" types has failed to produce a good therapeutic serum, when immunisation with the first has succeeded.

The Americans appear to have demonstrated sharp distinctions between their Types I, II and III, and to this extent there is a justification for their classification; but for the remaining strains it seems to have been found that typing is either impossible or can only be managed by making an indefinitely large number of groups. It would be unsafe to assume that these "atypical" strains are of relatively small importance; they are too numerous and too frequently responsible for cases of pneumonia.

The diversity of antigenic structure amongst strains of pneumococci presents a difficulty which has not yet been overcome. Though there is some occasional evidence of immunological relationship between strains which present serological differences, this is no proof that such differences are negligible; some of them certainly appear to be important and possibly all of them are; at all events it has not been found possible to discriminate between those which are important and those which are not.

Both Preston Kyes and the French school hope that they have found ways out of these difficulties, but their claims are in need of further confirmation.

Conclusions.

1. Results obtained from a continuation of the Rockefeller line of investigation will be awaited with interest; at present, there appear to be obstacles which render further progress difficult.

2. At the same time, other methods of attempting to make therapeutically useful antipneumococcal sera are worth considering, as it cannot be taken as proved that the Rockefeller method is the only one by which it is possible to attain success.

3. With certain parasitic bacteria, of which anthrax and fowl cholera appear to be examples, therapeutically potent antisera have been obtained, although the mechanism of their action is not clearly understood. It is just possible that a comparative study of the serological reactions of one of these bacteria and the pneumococcus might give some clue to an improved method of preparing antipneumococcal sera.

4. The study of recent work on pneumococci raises many difficult questions concerning the underlying principles of immunity reactions. A reconsideration of some of these general principles is necessary as an adjunct to experimental enquiry.

II.—TYPES OF PNEUMOCOCCI OBTAINED FROM CASES OF LOBAR PNEUMONIA.

By Fred. Griffith, M.B.

Introduction.

The study of types of pneumococci is important from two aspects, the therapeutic and the epidemiological. In the first place, almost all the available laboratory data show that the action of antipneumococcal serum is directed almost solely against pneumococci of the same type as that producing the serum. The American experiments on monkeys are an important part of this evidence and are supported by their observations on the selective action of antipneumococcal serum in the treatment of human pneumonia

On the epidemiological side, the close scrutiny of the serological types occurring in different bacterial species, such as the pneumococcus, meningococcus, etc., may some day provide an explanation why a ubiquitous and apparently harmless organism may suddenly become pathogenic for its host and of such high infectivity as to propagate an epidemic. The importance of the subject has recently been emphasised by the Health Committee of the League of Nations in their Conference

held in London on December 12-14, 1921. "It is very desirable," to quote from the memorandum submitted to the Conference by Dr. Madsen, "to obtain information regarding the occurrence "of different types in different countries in order that the thera- "peutic effect of pneumococcus serum may be properly deter- "mined. It is proposed, therefore, to start such an investigation "by inviting as many institutions as possible to examine strains "of pneumococci derived from pathogenic cases according to a "uniform plan."

In a preliminary *note on the serological types of pneumococci occurring in 100 consecutive cases of lobar pneumonia, derived mainly from the London area, I showed that the American Types I, II and III were demonstrable in approximately the same proportions as were found in the United States by the workers of the Rockefeller Institute. The report which I have now prepared for the Ministry of Health deals with a more extended inquiry into the incidence of the various types of pneumococci, together with my observations on the technique in the diagnosis of type and on the question of the immunological independence of types. Standard type strains and sera, received direct from the Rockefeller Institute, formed the starting point of this work on pneumococci.

I am much indebted to Dr. R. C. Harkness, Dr. C. Spurrell and many other medical officers of institutions for material used in this investigation.

Technique for the Differentiation of Type.

Isolation of Pneumococci.

The material received from the majority of the cases was sputum; in a small proportion pieces of consolidated lung tissue were examined. About 0·5 c.c. of sputum (or lung emulsion) was injected intraperitoneally into a mouse and the pneumococcus was grown from the heart's blood. The first culture was made direct from the blood into blood broth medium consisting of a mixture of equal parts of defibrinated rabbit blood and trypsin broth. At the same time a blood agar plate was sown from which colonies were selected if, as occasionally happened, the first culture was contaminated with other infective organisms from the mouse's blood; the influenza bacillus frequently multiplied in the blood of the first mouse but died out in subsequent passages through the mouse. An attempt was always made to avoid growing the strain on solid media in order not to weaken the virulence. Latterly, however, a number of single colony cultures have been tested from each strain, as it was found that the heart's blood of the mouse sometimes contained more than one type of pneumococcus. Occasionally, also, cultures were grown direct from the sputum as well as through the mouse; in the former case colonies were picked from blood agar plates. The spleen

* *Lancet*, July 30th, 1921, p. 226.

of the mouse, preserved by Heim's method, provided a reserve in the case of each strain.

Preparation of Pneumococcal Agglutinating Sera in Rabbits.

Adult rabbits were immunised with ascending doses of pneumococci which at first were killed by heat and finally were used living. The following are the details of a successful immunisation experiment with the American Type III strain on a rabbit, the serum of which developed an unusually high agglutination titre and protective value. The preliminary treatment with killed culture, *i.e.*, the centrifuged deposit of trypsinised broth culture heated to 60° C for ½ hour, lasted for three weeks, during which the animal received three series of intravenous inoculations. After an interval of six days immunisation was continued with living culture. This was first administered subcutaneously in a series of inoculations on four successive days followed, after a rest of seven days, by a second series of three inoculations. The third series was given intravenously, beginning with a dose of 0·5 c.c. of broth culture. The dose was gradually increased, the rabbit finally receiving the deposit of 20 c.c. of virulent broth culture. After 10 days from the last injection the rabbit was bled, the total period of treatment being 9 weeks. The fellow rabbit to the above received identical treatment, but it never developed a usable serum though immunisation was prolonged for more than twelve months. Probably peculiarities in individual rabbits are more important than the method of inoculation; some rabbits appear to be incapable of producing a good antiserum. As regards the condition of the antigen the use of living cultures guards against unfavourable changes. There is, however, the disadvantage that in spite of a high content of protective substances in the serum, induced by the preliminary inoculations of killed culture, the animals may die suddenly from endocarditis, large vegetations being found post-mortem on the mitral valve cusps. The risk may be avoided, since, as I have found more recently, active agglutinating and protective sera can be obtained from rabbits inoculated solely with culture killed by heat. Probably the maintenance of the strain in a condition of maximum virulence is an important factor in the production of a good immune serum, the fact of its being used living or dead being subsidiary. On this hypothesis every effort has been made to maintain the original virulence of those strains which have been used for the preparation of sera. The stock cultures have been passed through mice every two or three weeks and the culture in blood broth made direct from the mouse's blood has been kept on ice after a single night's incubation at 37° C.

Agglutinating sera of a sufficiently high titre for diagnostic purposes have been produced after two to six months treatment; the titre of these sera has ranged from 1 in 40 to 1 in 640 and occasionally has been as high as 1 in 1,280. In the case of certain strains it was very difficult to obtain any but relatively low titred sera.

Method of Performing Agglutination Test.

At the beginning of this investigation I used for agglutination tests pneumococci obtained by centrifuging glucose broth cultures and suspending the deposit in salt solution. Such suspensions, in my experience, are often unstable and may show some clumping with sera prepared with heterologous types. The tendency towards auto-agglutination is increased if the glucose broth cultures are incubated long enough for autolysis of the cocci to begin. Growth in the above medium should not be allowed to proceed beyond the stage where the broth culture is

still translucent, usually from 6 to 8 hours after a rich inoculation of a previously warmed broth. As a result of comparative tests I selected whole broth cultures as the most satisfactory suspension, and they have been used as a routine for some time. The broth in which the pneumococci are grown is Douglas's trypsinised broth*; the method of preparation differs from the original formula summarised below in that no glucose or calcium chloride are added and ordinary ox muscle is used in place of heart.

Method of Preparation of Douglas's Trypsin Broth.

Cut off the fat and large vessels from an average sized fresh bullock's heart: mince, add 4 litres of water, make faintly alkaline to litmus and heat to between 70° and 80° C. After cooling to 45° C., 1 per cent. of liquor trypsinæ co. (Allen & Hanbury) is added, *i.e.*, 40 c.c. to the four litres. Incubate at 37° C. for about 3 hours, then boil for ½ hour after slightly acidifying with acetic acid. Strain through muslin and adjust the reaction by the addition of dekanormal caustic soda solution to + 17 per litre (Eyre's scale). Add 0·13 per cent. calcium chloride and 0·25 per cent. sodium chloride: heat to boiling point and filter. Steam for 30 minutes on each of three consecutive days.

The reaction† of the broth should be carefully standardised, PH 7·8 is a favourable point, and the cultures should be incubated for 18 to 24 hours. Different strains produce very uniform growths, so that there is no necessity to estimate density, and there is rarely any tendency to spontaneous precipitation. The precipitate produced by the interaction of serum and whole broth culture is less bulky than with saline suspensions, but it is limited to the homologous type serum and does not occur with heterologous type sera. (After standing overnight the latter sera may produce at the most fine granules just visible to the naked eye). Thus the separation of pneumococci into serological types is emphasised, while the evidence of common relationship which can be obtained with more sensitive suspensions is not brought into undue prominence. In testing the type of a pneumococcus culture in the above way, it has never been necessary to titrate the agglutinating sera, the result being either positive or negative, and the sera have been used throughout in a dilution of 1 in 10, which is mixed with equal parts of broth culture.

When a type reaction occurs it is quite definite. Almost immediately after addition of culture to serum there is produced slight turbidity of the translucent broth culture. After incubation at 50° C. for one hour clumps form which increase rapidly in size with gentle shaking. The clumps aggregate into a compact mass after standing overnight and cannot be broken up by shaking. To obtain confirmation of a type reaction when the reaction with culture is not strong, it is useful to infect a mouse intraperitoneally with the culture and after death to wash out the peritoneal cavity with salt solution. Freed from

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* *Lancet*, 10th October, 1914, p. 891.

† Medical Research Committee. *Special Report Series*, No. 35, 1919.

fibrin and blood cells by centrifuging, these washings invariably give a strong reaction with the homologous type serum, provided the pneumococci have grown well in the peritoneal cavity.

Preparation and Test of Protective Sera.

The sera prepared against Types I, II and III were made with heated culture followed, in my earlier experiments, by living virulent culture, as I thought that the latter might be necessary for the production of protective substances. It was found, however, in the course of the preparation of immune sera against the strains not conforming to the three main types, that sera of considerable protective potency could be produced in rabbits solely by the inoculation of virulent culture killed by heat. Consequently, the technique ultimately adopted was the same, in all respects, as that described for preparing agglutinating sera, and it was found that a serum with a high agglutination titre was generally serviceable as a protective serum.

The protective power of the sera was tested on mice and the method was, in general, that first used by Neufeld. In this a fixed quantity of serum is used and the test is designed to determine the largest amount of broth culture against which this quantity of serum will protect the mouse from death, the period of observation being usually limited to 4 days.

So long as sufficient serum is used, the quantity is not a matter of importance and the dose of 0·2 c.c. used by Neufeld has been continued by most investigators, including the American. The serum is always tested for its prophylactic and not for its curative potency, but there is some difference of practice in regard to the length of the interval between the injection of the serum and the test culture. The method which I have adopted has proved satisfactory and has certain advantages in point of view of convenience; the serum is administered about 4 o'clock in the afternoon and the test culture between 10 and 1 o'clock the next day. Both serum and culture are injected intraperitoneally. It may perhaps be useful to describe some of the technical details of a protection experiment on mice.

Serum.—The serum is diluted 1 in 2·5 with broth so that 0·2 c.c., the prophylactic dose, is contained in 0·5 c.c.

Culture.—A pneumococcus culture is grown for 18 to 24 hours, in 0·5 c.c. to 1 c.c. of blood broth containing 50 per cent. of fresh whipped rabbit blood. The culture is well shaken before the quantity necessary for the first dilution is withdrawn. Virulence tests give more regular results with this medium than with plain broth; the diplococci remain single and all stain uniformly well with Gram.

The dilutions of culture are made as follows:—a series of six-inch test-tubes is filled with broth, the first containing 4·9 c.c. and each of the rest 4·5 c.c. To the first tube 0·1 c.c. of the blood broth culture is added and the mixture is well shaken. From the first tube, now containing 1 in 50 dilution of culture, 0·5 c.c. is taken over to the second tube to make a dilution of 1 in 500 and so on to the end of the series. A separate pipette is used for each dilution. The test dose is given in a total bulk of 0·5 c.c.;

thus 0·5 c.c. of the 1 in 50 dilution contains a dose of 0·01 c.c of broth culture.

Injection.—The serum, as well as the culture, is administered by means of a glass tube graduated to deliver 0·5 c.c., one end of which is drawn out to take the needle. A firm union is made between glass and needle by means of a piece of rubber valve tubing on which the mount of the needle fits. Delivery is controlled by the mouth through a long piece of rubber tubing which fits over the wool plugged end of the glass tubing.

P. M. Examination.—After the death of a mouse the blood is examined microscopically to confirm the existence of pneumococcus septicæmia, and if there is any doubt as to the cause of death, plate cultures are made from the blood.

The chief difficulty in the performance of protection tests is the maintenance of test cultures of standard virulence. Many of the strains of Group IV could not be used for protection tests, as it was impossible to increase the virulence up to the necessary degree. Protection experiments with strains of intermediate or irregular virulence may give misleading results. To bring the test culture up to maximum virulence it was invariably passed through a mouse the day before the test and the culture from the heart's blood served as the test culture.

Uniformity of Technique for the Diagnosis of Type.

The study of the serological types of pneumococci has made definite progress during the last few years and one may consider whether the time has now arrived when the adoption of uniformity of diagnostic methods may safely be recommended. The advantages of a uniform technique for the comparison of the results of different workers are obvious, but premature standardisation would lead to the stereotyping of a procedure which had not passed the test of prolonged general trial. This disadvantage might be obviated if each observer would control the results of the standard test by his own method and would publish any discrepancy between the two. The apparent divergencies, for example, between the results of the Pasteur and Rockefeller Institutes* can only be cleared up by the adoption of uniformity in technique and comparison of methods as advocated above. Until then it will be impossible either to obtain exact information upon the distribution of the three fixed types in different countries, or to make progress towards a useful classification of the so-called atypical strains of pneumococci which are found less commonly than the fixed types in cases of lobar pneumonia.

I therefore make, for the consideration of other workers in this field, the following suggestions towards the formulation of a uniform diagnostic test. In the first place a standard technique should be based on the methods of the Rockefeller Institute, the results of which have been confirmed in many countries;

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* A sample of the Pasteur Institute Type II serum (horse) which Dr. Truche kindly sent me agglutinated the Rockefeller Type I in my possession but not the Type II. Protection by the above serum was conferred on mice against both types, but more strongly against Type I than against Type II.

this implies that the standard test should be in accordance with the orthodox agglutination test. The question of the isolation of the pneumococcus, whether direct from the sputum or through the mouse, is debatable. For my own part I think it is preferable, wherever experiments on animals can be performed, to inoculate the sputum intraperitoneally into a mouse and to recover the pneumococcus from the heart's blood. It is a simpler method and yields more positive results than direct plating. The peritoneal washings provide a ready and certain means of diagnosing the type. It has been shown that more than one type of pneumococcus may occur at the same time in a sputum; the predominant virulent type, which will multiply in the mouse, is most likely to be the cause of the disease in the human being. With regard to certain technical details in the agglutination test, my own experience indicates that (1) the suspensions should be whole broth cultures, since they give the sharpest results; (2) agglutinating sera should be prepared in the rabbit, since this animal yields the most specific sera; (3) strains used for immunisation should be maintained in virulence and should therefore not be subcultivated for long periods on solid media.

Distribution of Types in the Present Series of 150 Cases.

The following figures give the percentage occurrence of the American types of pneumococci in the 150 cases of lobar pneumonia. In order to ascertain whether these figures are a sufficiently large sample to represent the normal incidence of the various types in the period, area, and circumstances of the inquiry, the results are analysed to show the relative proportions in each set of 50. For the purpose of comparison with the original American figures, those strains which did not react with any serum of the three "fixed" types are classed provisionally as "Type IV." The question of the American atypical Type II strains which I have included in the "Type IV" group will be referred to later.

	Types.				Period of Collection.
	I.	II.	III.	IV.	
1st 50 - -	11	17	6	16	April 1920—Nov. 1920.
2nd 50 -	14	20	1	15	Nov. 1920—May 1921.
3rd 50 - -	21	12	3	14	May 1921—Jan. 1922.
Percentage of total cases.	30·6	32·7	6·7	30	April 1920—Jan. 1922.

It will be seen that the above results are in fairly close agreement with the American results. They found* in 454 cases

* *Monograph of the Rockefeller Institute for Medical Research*, 16th October, 1917.

of lobar pneumonia—Type I 33·3 per cent. Type II 29·3 per cent. Type III 13 per cent. Type IV 20·3 per cent. and Atypical Type II 4·2 per cent.

Atypical Pneumococci.

As will be seen from the above table, 45 strains of pneumococci from cases of lobar pneumonia failed to agglutinate with any of the type sera I, II and III Such strains which did not conform to any of the three chief types were at first classed by the American workers as "Type IV" and it was thought that they were all serologically unrelated. In their later investigations these atypical strains were found to include a number of serological types, some of which could be shown by the use of concentrated Type II serum to be related to that type. These latter strains were therefore designated atypical Type IIA, IIB, etc. I have obtained from the Rockefeller Institute examples of types IIA and IIB and have prepared sera from them. As will be seen below, these two types appear to occur in cases of lobar pneumonia in this country next in order of frequency to the three fixed types. When moderate dilutions of strong agglutinating sera are used, there is no cross agglutination between the strains of Types II, IIA and IIB and their respective sera; they appear to be quite distinct serologically. Moreover Type II serum does not confer protection on mice against the atypical Type II strains. The evidence of the relationship that can be demonstrated by the use of concentrated Type II serum is of doubtful significance. For the present, therefore, I propose to include all the "atypical II" strains in the same category as the American heterogeneous "Type IV" and to term them collectively Group IV. In order to facilitate comparison with the results of other workers I have retained the designations, types IIA and IIB.

I have prepared agglutinating sera from a number of the atypical strains, or strains of Group IV with the object of attempting a serological classification of the group. After a preliminary test with these sera it was found that, while some of the strains used were of the same type, many of the Group IV pneumococci failed to agglutinate with any of the sera so far prepared. A further selection from the non-reacting strains was made and sera were produced with them. So far I have obtained sera of a sufficiently high agglutinating titre with 12 serologically different types of Group IV (including the American Types IIA and IIB).

The American workers have shown that the atypical strains occur frequently in the normal mouth and in respiratory conditions other than lobar pneumonia, whereas Types I and II are rare in such circumstances. During the present inquiry into the types of pneumococci occurring in lobar pneumonia, a number (40) of strains were obtained from the sputum of other cases of disease, principally of respiratory origin, *e.g.*, broncho-pneumonia, influenzal pneumonia, influenza, bronchitis, coryza, &c. From certain of these, selected on account of their high virulence for mice, sera were prepared which have been used to test the Group IV pneumococci from cases of lobar pneumonia. In the case of each of such strains at

least one homologue has been found in a case of lobar pneumonia. I think, therefore, it will be of interest and will obviate repetition, if the result of the serological classification of the 45 Group IV pneumococci from lobar pneumonia and the 40 strains from other diseases are considered together: 32 of the latter were Group IV, 5 Type III, 2 Type I and 1 Type II.

The following tables show the results of agglutination tests with 12 type sera, upon 77 strains of Group IV pneumococci. The strains used for immunisation are included in the "Totals" except in the two instances where these cultures were obtained from the Rockefeller Institute.

SEROLOGICAL TYPES DISTINGUISHED IN GROUP IV.

Type.	Homologues.	Source.	Total.
IIA. (Rockefeller Institute).	Pn. 101	Broncho-pneumonia	8 *cases*.
	,, 29	Lobar pneumonia, empyema	7 lobar pneumonia.
	,, 44	Lobar pneumonia	1 broncho- ,,
	,, 65	,, ,,	
	,, 69	,, ,,	
	,, 93	,, ,,	
	,, 154	,, ,,	
	,, 117	,, ,,	
IIB. (Rockefeller Institute).	Pn. 18	Lobar pneumonia, recurrent	12 *cases*.
	,, 73	Lobar pneumonia	9 lobar penumonia.
	,, 90	,, ,,	3 other diseases.
	,, 107	,, ,,	
	,, 132	,, ,,	
	,, 40	,, ,,	
†	,, 152	,, ,,	
	,, 159	,, ,,	
*	,, 1	Broncho-pneumonia.	
	,, 32	Necrosis of lung, secondary	
	,, 95	Scarlet fever.	
	,, 198	Lobar pneumonia.	
Pn. 10. Acute bronchitis.	Pn. 30	Lobar pneumonia	5 *cases*.
	,, 46	,, ,,	3 lobar pneumonia.
	,, 111	Broncho- ,,	1 broncho- ,,
†	,, 152	Lobar ,,	1 bronchitis.
Pn. 85. Lobar pneumonia.	Pn. 22	Lobar pneumonia	5 *cases*.
	,, 150	,, ,,	3 lobar pneumonia.
	,, 11	Acute bronchitis	1 acute bronchitis.
	,, 183	Influenza	1 influenza.
Pn. 42. Lobar pneumonia.	Pn. 13	Lobar pneumonia	3 *cases*.
	,, 89	Pleurisy and bronchitis	2 lobar pneumonia.
			1 pleurisy and bronchitis.

† * Two types in the same sputum.

Type.	Homologues.	Source.	Total.
Pn. 26 Broncho-pneumonia.	Pn. 9 „ 59	Acute bronchitis Lobar pneumonia	3 *cases.* 1 lobar pneumonia. 1 broncho- „ 1 bronchitis.
Pn. 41. Lung tuberculosis.	Pn. 57	Lobar pneumonia	2 *cases.* 1 lobar pneumonia. 1 tuberculosis of lung.
Pn. 70. Lobar pneumonia.	Pn. 24	Lobar pneumonia	2 *cases.* Both lobar pneumonia.
Pn. 87. Lobar pneumonia.	Pn. 32	Lobar pneumonia	2 *cases.* Lobar pneumonia.
Pn. 98. Lobar pneumonia.	Pn. 155	Acute coryza	2 *cases.* 1 lobar pneumonia. 1 coryza.
Pn. 84. Lobar pneumonia.	None	——	1 *case.* Lobar pneumonia.
Pn. 160. Lobar pneumonia.	Pn. 109 „ 166 „ 189	Lobar pneumonia Bronchitis Broncho-pneumonia	4 *cases.* 2 lobar pneumonia. 1 bronchitis 1 broncho-pneumonia.

Thus, 49 strains of pneumococci from 47 cases of respiratory disease (34 of which were from lobar pneumonia and 15 from other diseases) fall into 12 distinct serological types : 20 strains belong to the American types IIA and IIB, which are the predominating types in what I propose to call Group IV; and in one type, Pn. 84, there is only a single representative, namely. that from which the serum was prepared.

In two cases it will be noticed that two different types were obtained at different stages of the disease. From case 32 a pneumococcus of Type Pn. 87 was obtained from the sputum during the acute stage of pneumonia : a month later the patient died and at the post-mortem examination there was found necrosis of the lung, with cavity formation, from which Type IIB was obtained.

The second case, No. 152, was in the sixth day of lobar pneumonia when the sputum was examined and yielded Type Pn. 10. On the 12th day of the disease, and again on the 19th, a strain of IIB was obtained in pure culture from the sputum through the mouse : four colonies were examined from the 12th day specimen and 14 colonies from the 19th day and were all Type IIB. This question of different types of pneumococci from the same sputum is discussed later.

The remaining 28 strains of Group IV, 11 from lobar pneumonia and 17 from other respiratory diseases, reacted with none of the 12 Group IV type sera, and include therefore an unknown number of serological types.

The strains of Group IV are probably the common inhabitants of the nose and mouth, but they are evidently capable of producing both lobar and broncho-pneumonia, as well as other pathological conditions. On the other hand, although my observations are limited, I have so far rarely found Types I and II in any other condition than lobar pneumonia. A Type I strain was found in the cerebro-spinal fluid of a fatal case of meningitis, and in two cases of empyema Type II strains were obtained. During the influenza outbreak, January 1922, Type I was recovered twice and Type II once from cases of influenzal pneumonia.

The American observers have shown that Types I and II are of rare occurrence in the non-contact normal nose and mouth, and do not generally persist very long in the sputum after convalescence from lobar pneumonia due to one or other of them.

Type III more nearly resembles the strains of Group IV in its distribution. It is found in the normal mouth, though perhaps more frequently in some communities than in others. I may give an example of my own experience. At a school where there was an outbreak of influenza, out of 25 post-nasal swabs from schoolboys, 9 yielded pneumococci of Type III. In the present investigation, Type III has been found in 10 out of 150 cases of lobar pneumonia, in 5 out of 30 other infections (bronchitis or broncho-pneumonia); in 4 of the above cases (viz., 2 lobar pneumonia, 1 broncho-pneumonia and 1 acute bronchitis), it was found in association with pneumococci of Group IV.

Suggestions for Uniformity in Classification.

There is now sufficient accumulation of evidence to establish the three chief Types, I, II and III, defined by the workers of the Rockefeller Institute, as standard types for international purposes. At some future time the question of the nomenclature of the various types of pneumococci which differ from the three chief types will, no doubt, be considered by the Health Committee of the League of Nations, if, as is desirable, the results of workers in different countries are to be co-ordinated. In their pioneer work the American investigators classed these heterogeneous strains together as "Type IV." Later, they differentiated a number of types which reacted to concentrated Type II serum and designated them IIa, IIb, IIx, &c. In addition, Olmstead* has shown that the majority of the remaining "Type IV" strains in his collection could be classified into serological types. A comparison of the distribution of these

* *Journ. Immunology*, ii, p. 425. 1917.

"atypical" strains occurring in lobar pneumonia in different countries would, I think, be an important contribution to the study of pneumococci from the epidemiological aspect, and for this purpose uniformity of classification, as mentioned above, is essential. I have suggested that these heterogeneous strains might collectively be designated Group IV, each serological type being distinguished by a letter of the alphabet, *e.g.*, Group IVA, &c. The relationship of the "atypical" strains to the three chief types is a subject for future research. Too much emphasis should not, at the present stage, be laid on the atypical Type II strains as having relationship to Type II, but in order to avoid loss of continuity the letters A, B, X might be reserved for those strains of Group IV which correspond to the American atypical types, IIA, IIB, IIX.

Immunological Independence of the Races of Pneumococci.

In my preliminary note on the incidence of pneumococci in lobar pneumonia, I expressed the opinion that the results of my agglutination tests confirmed the American view, that the three types of pneumococci, I, II and III, were serologically independent. The results of my subsequent work are in accordance with this view. I now consider the evidence based on protection experiments on mice, and, in addition, give the results of agglutination and protection tests with the serological types of Group IV.

Protection Tests with Types I, II and III.

In the following experiment evidence of cross-protection was sought between Types I and II strains and sera. The virulence of the strains was such that they killed the control mice in two days after the inoculation of 0·000 000 1 c.c. of broth culture; the test culture was inoculated 19 hours after the injection of serum.

Serum II.	Test culture II.	Result.
0·2 c.c.	0·1 c.c.	Survived.
0·15 c.c.	0·1 c.c.	,,
0·1 c.c.	0·1 c.c.	,,
0·05 c.c.	0·1 c.c.	Died 3 days.
0·01 c.c.	0·1 c.c.	Died 1 day.

Serum II.	Test culture I.	Result.
0·2 c.c.	0·000 1 c.c.	Died 2 days.
0·2 c.c.	0·000 01 c.c.	,,
0·2 c.c.	0·000 001 c.c.	,,

Serum I.	Test culture I.	Result.
0·2 c.c.	0·1 c.c.	Survived.
0·15 c.c.	0·1 c.c.	,,
0·1 c.c.	0·1 c.c.	,,
0·05 c.c.	0·1 c.c.	,,
0·01 c.c.	0·1 c.c.	,,

Serum I.	Test culture II.	Result.
0·2 c.c.	0·000 1 c.c.	Died 1 day.
0·2 c.c.	0·000 01 c.c.	,,
0·2 c.c.	0·000 001 c.c.	,,

It is evident that, while both the Types I and II sera were highly protective for mice against the homologous strains, Type I serum exercised no protective power against Type II, nor Type II serum against Type I.

In another experiment, designed to determine whether the simultaneous inoculation of protective serum and living homologous culture would raise the animal's resistance against a heterologous type, the result was negative: the animals died like the untreated controls.

The details of the experiment are as follows:—A mixture was made of Type I serum and culture, 0·15 c.c. of serum to 0·01 c.c. of culture for a dose, and was inoculated into the peritoneal cavity of mice: the serum was sufficient to protect a mouse against 10 times the amount of culture. The next day each mouse which had received the mixture was injected with small doses, 0·000 1 and 0·000 01 c.c., of Type II culture. All the mice died of pneumococcal septicæmia. An exactly similar experiment with identical results was performed with Type II serum and culture, followed the next day by small test doses of Type I.

In a further experiment, mice were immunised by a preliminary dose of Type I serum, which was followed by small doses of a mixture of Types I and II culture. All the mice succumbed. The result was the same when the mice received an immunising dose of Type II serum.

Each of these three experimental tests indicates clearly that the protective action of such immune sera is very strictly confined to the homologous types of infection. Administration of a Type I serum has not the least inhibitory effect on an infection with Type II, and vice-versa. The same I have found to be true of Type III serum as regards the other two types, I and II.

Agglutination of Group IV Strains.

The various races of pneumococci resemble each other so closely in appearance of colonies and in the characteristic of bile solubility that there can be no doubt that they belong to one

species. The almost complete absence of any evidence of inter-relationship by the ordinary agglutination test is so much the more remarkable. It is true, as mentioned earlier, that the American observers find that certain of the types in Group IV are related to Type II, but, since this relationship can only be demonstrated by using equal parts of serum and suspension, its importance is doubtful. Again, as mentioned on page 22, when highly agglutinable suspensions of cocci in salt solution are used, there is often a precipitation in the form of fine clumps by low dilutions of heterologous sera. This does not occur when the suspensions consist of whole broth cultures of pneumococci.

The 12 type strains, which have been identified in Group IV, have been repeatedly tested against sera prepared from seven of their number and from the American strains I, II, III, IIa and IIb, equal parts of 1 in 10 serum and broth culture being used. Excepting in the case of *Pn. 26 and Pn. 42, where each of the two strains gave slight agglutination on a few occasions with the heterologous serum, there was a complete absence of any cross-agglutination; each strain reacted only with the homologous serum.

The results obtained with whole broth cultures have been confirmed by tests with the peritoneal washings from mice infected with the 12 types of Group IV. The agglutination tests, therefore, establish the serological independence of these Group IV strains from each other and from the three chief types.

Protection Tests with Group IV Pneumococci.

Experiments have been made to ascertain whether a preliminary inoculation of any of the three antipneumococcal sera of Types I, II or III confers protection on mice against a subsequent inoculation of a Group IV strain. The first two sera were protective against the homologous cultures (Type I and Type II), which were of high virulence; the Type III serum protected mice against Type III, but the strain used was not of maximum virulence. None of the three sera conferred appreciable protection on mice against any of the Group IV strains which were tested; even where the test strains were of moderate and irregular virulence, the mice died of pneumococcal septicæmia after some delay.

It has not been possible to attempt, in the case of the majority of the Group IV strains, an exact estimation of the presence of minor degrees of protective power in their sera against other members of the group, on account of their irregular

* An absorption of agglutinin test, made with Pn. 42 and Pn. 26 strains and sera, showed that the homologous strains removed the agglutinin, and the heterologous strains had no effect. In no other instance was the absorption of agglutinin test found to be necessary.

virulence. None of the sera, however, has conferred complete protection against any but the homologous strain, though sometimes mice have only succumbed to pneumococcal septicæmia 8 and 10 days after inoculation. This delay I am inclined to attribute not to specific protection, but to an increased general resistance induced by the intraperitoneal injection of serum protein.

A few strains, *e.g.*, Pn. 30, Pn. 46 and Pn. 10, which were of the same agglutination type, and Pn. 9, were almost equal in height and regularity of virulence to the Type I and Type II strains, and some protection experiments made with them have given satisfactory results. The test sera were inoculated in the evening, in doses of 0·2 c.c., and were followed by the test cultures the following morning. All the inoculations were made intraperitoneally, with broth diluted to 0·5 c.c.

TABLES SHOWING RESULTS OF PROTECTION TESTS *v.* PN. 30 AND PN. 46.

Serum (0·2 c.c.)	Test Culture.	Result.	Test Culture.	Result.
Pn. 46	Pn. 30 0·1 c.c. -	Survived	Pn. 46 0·1 c.c. -	Survived.
	,, 0·1 c.c. -	,,	,, 0·01 c.c. -	,,
	,, 0·01 c.c. -	,,	,, 0·001 c.c. -	,,
	,, 0·001 c.c. -	,,	,, 0·0001 c.c. -	,,
Pn. 10	Pn. 30 0·1 c.c. -	Survived	Pn. 46 0·1 c.c. -	Survived.
	,, 0·1 c.c. -	,,	,, 0·01 c.c. -	,,
	,, 0·01 c.c. -	,,	,, 0·001 c.c -	,,
	,, 0·001 c.c. -	,,	,, 0·0001 c.c. -	,,
Pn. 26	Pn. 30 0·000 01 c.c.	D. 2 days	Pn. 46 0·0001 c.c. -	D. 2 days.
	,, 0·000 001 c.c.	D. 2 days	,, 0·000 01 c.c.	D. 2 days.
	,, 0·000 0001c.c.	D. 2 days	,, 0·000 001 c.c.	D. 2 days.
Pn. 42	Pn. 30 0·000 01 c.c.	D. 2 days	Pn. 46 0·0001 c.c. -	D. 2 days.
	,, 0·000 001 c.c.	D. 2 days	,, 0·000 01 c.c.	D. 2 days
	,, 0·000 0001c.c.	D. 2 days	,, 0·000 001 c.c.	D. 2 days
Type I	Pn. 30 0·000 01 c.c.	D 2 days	Pn. 46 0·000 01 c.c.	D. 1 day.
	,, 0·000 001 c.c	D 2 days	,, 0·000 001 c c	D 2 days.
	,, 0·000 0001c.c.	D. 2 days	,, 0·000 0001c.c.	D. 2 days.
Type II	Pn. 30 0·000 01 c.c.	D. 2 days	Pn. 46 0·000 01 c.c.	D. 1 day.
	,, 0·000 001 c.c.	D. 2 days	,, 0·000 001 c.c.	D. 2 days.
	,, 0·000 0001c.c.	D. 2 days	,, 0·000 0001c.c.	D. 2 days.
Type III	Pn. 30 0·000 01 c.c.	D. 2 days	Pn. 46 0·000 01 c.c.	D. 2 days.
	,, 0·000 001 c.c.	D. 2 days	,, 0·000 001 c.c.	D. 1 day.
	,, 0·000 0001c.c.	D. 2 days	,, 0·000 0001c.c.	D. 2 days.

The control mice inoculated with Pn. 30 culture in doses of 0·000 001 c.c., 0·000 000 1 c.c. and 0·000 000 01 c.c. died in two days. A similar result was obtained with Pn. 46 culture.

PROTECTION TEST *v.* PN. 9.

Serum 0·2 c.c.	Test culture Pn. 9.	Result.
Pn. 26	0·1 c.c.	Survived.
	0·1 c.c.	„
	0·01 c.c.	„
	0·01 c.c.	„
	0·001 c.c.	„
Pn. 42	0·1 c.c.	Died 1 day.
	0·01 c.c.	„
	0·001 c.c.	„
	0·0001 c.c.	„
	0·000 01 c.c.	„
Pn. 10	0·000 1 c.c.	Died 1 day.
	0·000 01 c.c.	„
	0·000 001 c.c.	Died 2 days.
	0·000 000 1 c.c.	Died 6 days.
IIA	0·000 1 c.c.	Died 2 days.
	0·000 01 c.c.	Died 1 day.
	0·000 001 c.c.	„
	0·000 000 1 c.c.	Survived.

Pn. 26 and Pn. 9 were of the same agglutination types. Pn. 9 was highly virulent in this experiment, killing mice in a dose of 0·000 000 01 c.c. of broth culture in one day; this strain was subject to unexpected variation in virulence.

VARIETIES OF TYPE YIELDED BY THE SAME PATIENT.

Reference has already been made to the observation of the American workers on pneumococci, that Types I and II disappear from the sputum during or soon after convalescence from lobar pneumonia, and are often replaced by the types of pneumococci which have been found of common occurrence in the normal mouth. The American hypothesis is that the type strains die out. An alternative theory is that the virulence of Types I and II becomes attenuated during convalescence, and that this change is accompanied by mutation of type characters, which now become degraded into those of the heterogenous and less virulent group termed IV. There are two experimental difficulties about this view. Some of the IV strains are quite as virulent for mice as any of the I and II strains; so the characteristics of IV cannot be taken as evidence of loss of virulence, at any rate for the mouse. Again, I and II strains do not lose their agglutinability towards the homologous sera after prolonged subculture, though they may have become completely avirulent: it is not found, at any stage of subculture, that mutation of type has occurred in any of the colonies. Still, it is theoretically possible that mutation may occur in nature,

though it cannot be reproduced *in vitro*. It occurred to me that some information might be obtained on this point by the examination of strains grown from sputum during the acute stage of pneumonia and later during convalescence. If mutation occurred, one might expect some regularity in the serological characters of the strain which replaced the Types I and II. A number of experiments have been made on these lines in infections with Types I and II and I have made it the routine to test several colonies from each case, obtained either through the mouse or direct from the sputum. Although many of the infections have been apparently pure, it is very striking how often atypical bile-soluble diplococci can be found in sputum, even during the acute stage, together with the Types I and II to which must be attributed the production of the pneumonia. Before proceeding to describe some of these results, it may be useful to indicate the method adopted for investigating single colony cultures.

Method of obtaining Single Colony Cultures.

At first some difficulty was experienced in obtaining uniformly successful growth of colonies of pneumococci in broth. The following procedure was ultimately adopted. The material, sputum or mouse blood, was plated on nutrient agar, to which was added 5 per cent. of chloroformed blood cells from horse or ox with 5 per cent. filtered horse or bovine serum. This makes an opaque medium on which pneumococci grow well and form characteristic colonies, so that it was rare to select a colony which failed to pass the bile solubility test. The selected colony was touched with the point of a spatula and rubbed up in a small tube containing about 0·5 c.c. of 50 per cent. whipped rabbit blood in broth. After incubation overnight, a loopful of the deposited cells, dark in colour where growth of pneumococci had occurred, was transferred to a tube containing about 5 c.c. of trypsinised broth. After a night's incubation a peptone broth culture was inoculated from the tryp. broth (the latter being unsuitable for the bile solubility test), the remainder of which was used for test with agglutinating type sera.

Details of some Cases from which Mixed Cultures were obtained.

The following are a few examples of cases of pneumonia in which the examination of the sputum revealed the presence of more than one type of pneumococcus. It has been observed that a second type has more frequently been obtained in the cases where the primary infection was due to T. I than T. II. In no instance have both Types I and II been obtained from the same sputum. The second type has always belonged to Group IV, and it is of some interest to note that, where it has been possible to identify a homologue of the 12 types of Group IV,

it has more often belonged to types Pn. 42 and Pn. 26 than to any one other. This may mean no more than that these two are common types in the pharynx, but there is not at present sufficient evidence to decide whether this must be accepted as the explanation.

Case 1.—*Pn.* 112.

The material was the sputum from a man aged 45 years, and was taken on the sixth day from the onset of pneumonia (a crisis had not occurred). A mouse inoculated with the sputum died on the following day: the peritoneal washing, which contained a variety of organisms, was tested with the type sera and gave a slight positive reaction with Type I. The blood of the mouse was plated and six pneumococcus colonies were subcultivated. These were tested in whole broth cultures with the type sera. One colony culture reacted with Type I serum, two with Pn. 26 serum, one with Pn. 42 serum, and one with Pn. 87 serum. Thus, four distinct serological races were present in the sputum.

Case 2.—*Pn.* 118.

The sputum was from a boy aged 10 years, and was taken on the fourth day of pneumonia. The peritoneal washing from the inoculated mouse gave a Type I reaction, and eight colony cultures grown from the mouse's blood all reacted with Type I serum.

A second specimen was taken 13 days later, that is, 11 days after the crisis. The sputum was plated directly and eight pneumococcus colonies were tested with the type sera. Three colonies were Type I, two were Pn. 42 type, and three did not react with any of the sera. A mouse died after inoculation with the sputum, and its blood was plated: five colonies were tested, of which two were Pn. 42 type and three did not react with any of the sera. Type I was not obtained through the mouse.

A third specimen was taken 19 days after the crisis, on the 27th day of the disease. Twelve pneumococcus colonies, grown direct from the sputum, did not react with any of the sera. From the blood of the mouse inoculated with the sputum, 21 colony cultures were made: fourteen of these were Type I, three were Pn. 42 type, and four were negative to all the sera.

Case 3.—*Pn.* 106.

Sputum from a case of pneumonia on the fourth day of the disease (pre-critical) was inoculated into a mouse. The peritoneal washing from the infected mouse gave a reaction with Type II serum.

A second specimen of sputum was taken three weeks later. Five colonies were grown direct from the sputum, four of which were Type II, and one was Pn. 42 type. The blood of the inoculated mouse was plated, and 16 colonies were tested: 14 belonged to Type II and two were Pn. 42 type.

Case 4.—*Pn.* 125.

A specimen of sputum from lobar pneumonia in a man aged 18 years was taken before the crisis. A mouse was inoculated, and 13 colonies were grown from the blood. Seven reacted to Type I serum, five to Pn. 26 serum and one was negative with all the sera.

Case 5.—*Pn.* 67.

Sputum from a case of pneumonia on the eighth day of the disease in a man aged 44 years was inoculated into a mouse: the peritoneal washing reacted with Type I serum.

A second specimen of sputum was examined 16 days later during a relapse: a mouse was inoculated and its peritoneal washing reacted with Type III serum. A second mouse was inoculated with the dried spleen of the first mouse and on its death its blood was plated. Five pneumococcus colonies were tested, two of which reacted with Type III serum and three with Pn. 42 type.

The above are cases where the secondary pneumococci belonged to one or other of the types with which sera have been prepared. In some cases, strains, which have been proved bile soluble, have been found accompanying or replacing the Types I or II, and have not been serologically identified. In many of the tests when repeated examinations have been made, either the original infecting strain has survived in a pure state, or no pneumococci have been found.

For example a sputum (Pn. 120) from a man aged 22 years yielded eight colonies, all of which were Type II, on the sixth day of pneumonia: 11 days after the crisis, six colonies were all Type II: 19 days after the crisis, the sputum failed to infect a mouse.

In another case, Pn. 146, tested before the crisis, Type I was found in 12 colonies direct from the sputum and in 20 colonies through the mouse. The sputum taken eight days later failed to infect a mouse.

In a third case the sputum from a man aged 43 years yielded Type II on the third day of pneumonia. During convalescence, 16 days later, a second specimen was obtained which also yielded Type II only, 10 colonies direct from the sputum and 10 through the mouse being examined.

No decision can be made from these results on the relationship of the primary and secondary types of pneumococci in a sputum. They emphasise the necessity of caution in deciding on the type of infecting pneumococcus, at least where the material is not obtained direct from the diseased lung.

Action of Culture and Pneumococcal Exudates on Immune Serum.

The American investigators have shown that the precipitate, which results from mixing an immune serum with the culture autolysate of the homologous pneumococcus, contains the protective substances of the serum. The possibility of obtaining a therapeutic reagent in a concentrated form by this means was considered by them, but it was found that the product of the reaction was liable to be contaminated, and, moreover, the protective properties were unstable. I have made some experiments on the above lines, with a view to studying the mode of action of a serum which has the property of protecting mice against a subsequent inoculation of a virulent pneumococcus. Many views have been put forward, but that of Neufeld still holds the field, namely that the active substances in a protective serum are bacteriotropic, and are only available against a type of pneumococcus similar to that which produced the serum. The results of the absorption tests given on page 41, in which treatment with the homologous pneumococcus removes the protective power of the serum, afford some confirmation for this view. There is, however, the possibility that the protective substances are carried down with the agglutinated cocci, and do not actually adhere to them.

The following experiment shows that a washed heated suspension of pneumococci precipitates the protective substances from a serum without impairing their properties.

Type I serum was treated three times with washed heated suspensions of Type I culture. The resulting deposit was removed by centrifuging and was washed with broth. The supernatant fluid and the washed deposit were tested separately in mice: the latter still protected mice against 0·01 c.c. of culture, the former failed to protect.

A similar experiment was made with the serum of Pn. 10, a Group IV strain.

The heated deposit of broth culture was added to the serum on three successive occasions, and the mixture was then centrifuged. The fluid remained a little opalescent in spite of the prolonged centrifuging, but after standing overnight small clumps formed which settled, leaving the fluid quite clear. The deposit was washed twice and was suspended in a quantity of salt solution equal to the original serum.

The deposit and the original supernatant fluid were each tested upon mice for protective properties. The deposit protected mice against 0·01 c.c. of culture but failed to protect against 0·1 c.c. The supernatant fluid protected against 0·000 01 c.c. but not against 0·000 1 c.c.

The experiment was repeated with the same serum and a smaller amount of culture added on one occasion only. The supernatant fluid protected against 0·01 c.c. of culture but not against 0·1 c.c. The deposit failed to protect against 0·01 c.c. but protected against 0·000 1 c.c.

It will be seen that in the first experiment, where a large amount of absorbing culture was used, more of the protective substances were demonstrated in the deposit than in the supernatant fluid. The converse was the case in the second experiment, where the exhaustion of the serum was less severe, *i.e.*, more of the protective bodies remained in the supernatant fluid than were taken down with the centrifuged deposit. The effect of heating the deposit to 69° C. for 1 hour was tried, and it was found that the protective properties were not impaired.

Instead of culture the peritoneal washings of a guinea-pig which had been inoculated intraperitoneally with living culture of Type II were used to treat a Type II serum. It was thought that perhaps pneumococcal aggressins might be produced, and that these might neutralise the protective power of the serum. In effect the results were the same as when the serum was treated with culture cocci; the protective substances went down with the precipitate and still retained their active properties.

The details of the experiments include a few points of interest. Before addition to the serum the peritoneal washings were centrifuged clear, and were sterilised by heating to 60° C. followed by chloroform. After the mixture had stood over night, a firm gelatinous precipitate was formed. This was removed by centrifuging and the supernatant fluid was treated for the second time with the peritoneal washings: there was no further visible reaction.

The deposit was ground up in a mortar but it was impossible to make a uniform suspension in broth, as the particles almost immediately became agglutinated. An effort was made to inoculate mice with equal parts of the deposit by dividing it up into small pieces about the same size. Five mice were thus inoculated intraperitoneally, and test doses of a Type II culture, virulent for mice in doses of 0·000 000 01 c.c., were given. All five mice remained alive after doses ranging to 0·000 01 c.c. The supernatant fluid was still protective against 0·000 01 c.c. (1 out of 2 mice).

This experiment was repeated with Type II serum and peritoneal washings with similar results. Before treatment, the Type II serum in doses of 0·2 c.c. protected against 0·1 c.c. of Type II culture. The gelatinous deposit, representing approximately 0·2 c.c. of serum, protected against 0·01 c.c. of culture.

These experiments show that the washed cocci and the clear peritoneal washings each precipitate the protective substances from immune serum but without destroying their protective properties. Is this evidence that the substances are bacteriotropic in nature and form a loose combination with the absorbing cocci?

The following experiment appears to indicate that, if any combination occurs, it must be a very loose one.

A mixture was made of 1 c.c. of Type I serum and 0·01 c.c. of living Type I broth culture, and was allowed to stand in the ice chest overnight. The next day, the mixture was well shaken, and small amounts, representing a mixture of 0·01 c.c. of serum and 0·000 1 c.c. of culture down to 0·000 001 c.c. of serum, and 0·000 000 01 c.c. of culture, were inoculated into mice. All the mice survived. The remainder of the mixture was centrifuged, and the serum was poured off. Fresh serum was added to the deposit: after a few hours this was poured off, and the deposit was washed. The deposited cocci, which were now presumably sensitised, were resuspended in broth, and were inoculated into mice in doses estimated to contain from 0·000 001 c.c. to 0·000 1 c.c. of broth culture. One of three mice died of pneumococcal septicæmia, two survived.

The first part of the above experiment was repeated with the same amount of serum but ten times the amount of culture, *i.e.*, 1 c.c. serum to 0·1 c.c. culture. The mixture of serum and culture was inoculated into mice as before, and all the mice survived excepting the one which received the smallest amount of culture and of course the smallest amount of serum.

The above results would seem to indicate that the cocci were not sensitised, but that the mice which survived owed their protection to the serum inoculated along with the culture; in the case of the highest dilution of the mixture the amount of serum was inadequate to give any protection, even against the minute dose of culture.

The following experiment supports this view. The centrifuged deposit from the above mixture was washed with broth and was resuspended. All the mice died after inoculation with amounts estimated to contain from 0·01 c.c. to 0·000 001 c.c. of broth culture.

The action of culture on an immune serum in the animal body is apparently different from its action in the test-tube. It was found in the former that the effect of the intravenous inoculation of culture into an immune animal was to abolish temporarily the protective power of the serum. This circumstance may perhaps help to explain the occurrence of endocarditis, following the inoculation of living culture, in rabbits which have been highly immunised with killed culture. It has happened frequently, *see* p. 22, and with every type of pneumococcus, in spite of the fact that the serum of the rabbit was capable of conferring protection on mice. One can imagine that the temporary inhibition of protection, as a result of the inoculation, enables the pneumococci to establish themselves on the avascular cardiac valves.

Absorption of Protective Substances.

Several absorption experiments have been done with Types I and II sera, and it has been shown that, by treatment with the

respective homologous strains, both the agglutinins and protective substances are removed with the deposited cocci. For example, 3 c.c. of T. I serum were treated with the deposit of 200 c.c. of heated broth culture contained in 4 c.c. of broth: after the mixture had stood overnight in the ice-chest, it was centrifuged and the supernatant fluid was removed for inoculation into mice in doses of 0·5 c.c. (*i.e.*, equivalent to 0·2 c.c. of serum).

PROTECTION TEST WITH ABSORBED AND UNABSORBED TYPE I SERUM.

Type I serum unabsorbed.	Type I test culture.	Result.
0·2 c.c.	0·1 c.c.	Survived.
0·15 c.c.	0·1 c.c.	„
0·1 c.c.	0·1 c.c.	„
Type I serum absorbed.	**Type I test culture.**	**Result.**
0·2 c.c. (in 0·5)	0·01 c.c.	Died 2 days.
„ „	0·001 c.c.	„
„ „	0·000 1 c.c.	„
„ „	0·000 01 c.c.	„
„ „	0·000 001 c.c.	„
Normal serum.	**Type I test culture.**	**Result.**
0·3 c.c.	0·000 000 1 c.c.	Died 2 days.
0·3 c.c.	0·000 000 1 c.c.	„

The Type I culture was fully virulent, and control mice inoculated with 0·000 000 01 c.c. of broth culture died in 2 days.

The sera of Type I and Type II were fairly readily exhausted of their content of protective substances by treatment with the homologous strain. On the other hand greater difficulty has been experienced in removing the protective power from the particular Group IV sera which have been tested, in spite of the use of large quantities of the absorbing culture.

There are technical difficulties in the way of demonstrating complete exhaustion of a strong protective serum. As is well known, a single treatment of a concentrated serum with the homologous culture cannot remove the whole of the agglutinins and similar antibodies.

The serum cannot be diluted beyond a certain point, since it is not desirable to inoculate a larger amount of fluid than 0·5 c.c. or at the most 1·0 c.c. intraperitoneally into a mouse. With a dilution of 1 in 2·5, which has generally been used, the

equivalent of 0·2 c.c. of serum is contained in 0·5 c.c.; it has been found, however, that dilution to 1 in 5 does not affect the protective power of a serum.

The amount of absorbing culture used cannot exceed a certain limit: in some of the absorption experiments, the amount of thick culture emulsion added to pure serum was such that, after prolonged centrifuging, only a third of the original serum could be recovered.

Some examples of absorption experiments with Group IV sera will be given in the case of Pn. 10, the serum of which was strongly protective and agglutinating, while the cultures of Pn. 10 and Pn. 30 of the same type were highly virulent.

Experiment 1.—Pn. 10 serum was absorbed with Pn. 30 culture, which was not the homologous strain but was of the same type. The test of protection was also made with Pn. 30 culture. Before absorption, the serum inoculated in doses ranging from 0·2 c.c. to 0·05 c.c., protected mice against 0·1 c.c. of broth culture of the test strain, Pn. 30; 0·01 c.c. of serum was ineffective. After absorption, which consisted in treatment of 1·5 c.c. of serum with the deposit of 800 c.c. of broth culture on two successive occasions, the final dilution of serum being 1 in 5, there was some diminution in protective power but not complete exhaustion. The serum in doses of 0·2 c.c. still protected mice against 0·01 c.c. of broth culture but failed against 0·1 c.c.

Experiment 2.—To 1 c.c. of Pn. 10 serum was added the deposit of 400 c.c. of broth culture of Pn. 30, the final concentration of serum in the mixture being 1 in 5. After standing overnight in the ice-chest, the mixture was centrifuged. As before, the unabsorbed serum in doses of 0·2 c.c. protected mice against 0·1 c.c. of broth culture of the absorbing strain Pn. 30. After absorption 0·2 c.c. of serum protected against 0·001 c.c. but not against 0·01 c.c. In this instance removal of the protective bodies was more complete than in the first experiment.

Experiment 3.—Absorption of Pn. 10 serum with the homologous strain and test of protection versus Pn. 30 culture. Two cubic centimetres of serum were treated with the deposit of 300 c.c. of broth culture contained in 8 c.c. of uncentrifuged broth culture. The culture was added to the serum on two separate occasions and the mixture, in which the serum was diluted 1 in 10, was centrifuged after standing overnight in the ice-chest. The unabsorbed serum (0·2 c.c. in 2 c.c.) protected mice against 0·1 c.c. of culture. After absorption 0·2 c.c. failed to protect mice against 0·000 001 c.c. of broth culture. Thus almost complete exhaustion was effected.

The above experiments show that Pn. 10 serum was not readily exhausted of its protective substances. On the other hand a single treatment of 1·5 c.c. of Type I or Type II serum in a concentration of 1 in 5 with the deposit of 400 c.c. of broth culture resulted in effective absorption.

Experiment 4.—Another Group IV. serum, Pn. 26, was absorbed with a suspension of the homologous strain. To 2 c.c. of serum were added 2 c.c. of culture emulsion, after an interval, a second 2 c.c., then 1 c.c.; after standing overnight in the ice-chest the mixture was centrifuged and 4·5 c.c. of clear supernatant fluid were recovered out of 7 c.c., the remainder consisting of deposited culture. A further quantity of culture was added to the recovered dilution, which after a few hours was centrifuged clear. The absorbed serum was tested for protective power versus a culture of the same type which killed mice in doses of 0·000 000 01 c.c. of broth. It was found still to protect against 0·000 001 c.c. but failed against 0·000 1 c.c. and higher doses.

Effect of Intravenous Inoculation of Culture on the Protective Substances and Agglutinins in an Immunised Animal.

The subject of the experiment was a rabbit which had already been immunised against a pneumococcus of Group IV, but which had not been inoculated for two months. The object was to ascertain (1) the effect of re-inoculation of culture upon the antibodies present in the serum, and (2) the time after the re-inoculation at which these substances attained their maximum concentration. A specimen of the rabbit's serum was taken before the inoculation, which consisted of the deposited cocci from 100 c.c. of broth culture heated to 60° C. for ½ hour and administered intravenously at four short intervals. Samples of blood were taken the day after inoculation and on the 3rd, 5th, 9th and 11th days. The sera were preserved in the ice-chest until the final bleeding, and the tests of protection and agglutination with all the samples were made on the same day.

The serum before inoculation did not agglutinate in 1 in 5 dilution; it protected against 0·000 001 c.c. of broth culture of the homologous strain but not against 0·000 01 c.c.

The day after inoculation there was no agglutination and no protection. On the 3rd day there was still no agglutination demonstrated in 1 in 5 dilution, but the serum protected against 0·000 1 c.c. of culture (5 out of 6 mice) and 0·001 c.c. (1 out of 2 mice).

On the 5th day the serum contained agglutinins and protected against 0·01 c.c. of culture (3 mice), but there was some irregularity, as one or two of the mice inoculated with smaller quantities of culture died. The 9th and 11th day samples of serum protected against 0·01 c.c. of culture, but failed to protect against 0·1 c.c.; apparently the maximum protective power was attained on the 5th day.

The rabbit was given a rest of three weeks. At the end of this period its serum gave only a faint trace of agglutination and had lost some of its protective power, *e.g.*, 1 out of 5 mice survived a dose of 0·000 1 c.c. of culture and 3 out of 4 mice a dose of 0·000 01 c.c. The inoculation of heated culture deposit of 200 c.c. of broth culture was then repeated. After the re-inoculation agglutinins and protective substances were absent from the samples of serum collected on the 1st and 2nd days.

On the 5th day the agglutininins had reappeared and the serum again protected against 0·01 c.c. of culture (2 out of 4 mice) This titre was maintained on the 9th and 11th days, but in no instance was the serum found to be sufficiently strong to protect against 0·1 c.c. of culture.

In the course of the above protection tests there was noticeable a certain irregularity in protection, *e.g.*, a sample of serum protected some mice against 0·01 c.c. of culture but failed to protect every mouse against an inoculation of a smaller dose.

This irregularity does not generally occur in the case of a serum derived from an animal which has been strongly immunised by weekly series of inoculations; it may possibly be ascribed to the single inoculation.

After an interval of three months the weekly injections of heated culture were continued for six weeks: at the end of the treatment the agglutination titre was up to 1 in 160. The inoculation of living culture was then begun for the first time, 0.5 c.c. of broth culture being administered subcutaneously. The doses were increased up to 5 c.c. intravenously, when the animal died at the end of the 3rd week, six days after the last inoculation. The post-mortem examination revealed large vegetations on the cusps of the mitral valves, and diplococci were demonstrated in the blood.

The blood serum, collected while the animal was moribund, had an agglutination titre of 1 in 320, and in doses of 0·2 c.c. conferred protection on mice against 0·01 c.c. of broth culture of the homologous type, the fatal dose of which was 0·000 000 001 c.c. The interpretation of this result has been discussed on page 40.

Disappearance of Pneumococci in Passively Immune Mice.

Many careful and elaborate investigations have been made by others on the disappearance of pneumococci from protected mice with the object of explaining the mode of action of the immune serum. The following observations illustrate a few particular points and are in no way an attempt to deal exhaustively with the subject.

Mice were protected by the intraperitoneal injection of 0·2 c.c. of a Group IV serum. The following day, a number of the mice received the dose of 0·1 c.c. of the homologous culture given intraperitoneally. After an interval of one hour, a protected mouse and a control mouse, which had received culture only, were killed. Smear preparations from each of the two showed in the peritoneal cavity numerous pneumococci, which in the protected mouse were in clumps. Plate cultures also were made from the blood: from the unprotected mouse numerous colonies were grown, while the plate from the blood of the protected mouse remained sterile. The blood of the latter animal, however, did contain living pneumococci, since 0·5 c.c. of the blood sown in broth produced a positive culture. A second mouse was alive and well 48 hours later. It was killed, and cultures were made from the blood and peritoneal cavity; all proved negative.

Four of the remaining protected mice were inoculated subcutaneously with 0·1 c.c. of the homologous culture, in addition to several unprotected controls. After 4 hours, one of each series was killed; in both cases smear preparations showed the presence of capsulated diplococci at the seat of inoculation. A protected mouse, killed after 24 hours, still showed living pneumococci at the seat of inoculation, but the blood was

apparently sterile. After 48 hours no living pneumococci could be recovered from the lesion or from the blood.

The results shows that pneumococci do multiply at the seat of inoculation, but very few gain access to the blood stream and survive; capsule formation takes place in the protected mouse.

General Conclusions.

1. A uniform technique for diagnosis of types of pneumococci is desirable, and it should be based on the American methods, except that whole broth culture might be used for the suspension.

2. The American Types I, II and III are serologically distinct, and they occur in cases of lobar pneumonia in this country in about the same proportions as in the United States.

3. The American types, IIA and IIB, occur in cases of lobar pneumonia next in order of frequency to the Types I and II.

4. The American "Type IV" is responsible for cases of lobar pneumonia in this country somewhat more frequently than in America.

5. "Type IV," including those strains which appear to be related to Type II, such as the American IIA, IIB, &c., might be designated Group IV, since it contains a large number of separate types. These types might be distinguished by the letters of the alphabet: thus, types IIA and IIB would become Group IVA and Group IVB, and so on.

6. Protection tests on mice confirm the serological independence of the types of pneumococci.

7. Protective sera act equally well, whether inoculated subcutaneously or intraperitoneally, whether the test culture is injected immediately after the serum or after an interval of 18 hours. The test culture should be administered intraperitoneally to ensure accuracy of dosage.

8. Certain protection experiments are apparently not in agreement with the bacteriotropic theory of Neufeld.

9. The absorption experiments have shown that there is no firm union between antigen and antibody, and no neutralisation of the protective properties.

10. I am of the opinion that further work is necessary before a final conclusion can be drawn as to the mode of action of antipneumococcal serum.

III.—THE SIGNIFICANCE OF SEROLOGICAL DIFFERENCES AMONGST PNEUMOCOCCI.

By Arthur Eastwood, M.D.

Introduction.

Research on pneumococci is intimately concerned with a serological complication which has arisen from the discovery of antigenic differences amongst members of the species. By selective tests, in which concordant results are obtained with agglutination, precipitation, and protection for mice, a large number of different "serological races" has been demonstrated; in fact, the number of possible varieties appears to be so large as to make an inclusive classification impossible.

This is a matter of importance practically as well as theoretically, because it appears to involve difficulties in the preparation of therapeutic sera. If the main factor in the production of a useful serum were some specific substance common to all virulent pneumococci, one would expect an antiserum prepared from a single typical strain to be polyvalent in its protective and therapeutic action; and then minor antigenic differences might be disregarded. But this expectation has not been realised. On the contrary, there is a large accumulation of laboratory evidence in support of Neufeld's original contention that an immune serum is useful only against strains which are identical, in every antigenic respect, with the strain used for immunisation.

Hence one cannot dismiss this difficulty by merely offering the suggestion, which has been made about the "serological

races" of certain other bacterial species, that these serological differences are unimportant therapeutically, or that they are merely minor idiosyncrasies which needlessly distract attention from the identity of the strains in more essential biological functions, such as toxicity. Theoretically, it may be reasonable to argue that antigenic differences are not all of equal importance. A protein compound may possess a large number of special chemical groups, and the particular configuration of one of these groups may determine whether it is acted upon by a certain enzyme or not; but the configuration of the other special groups may be of no importance to the enzyme. Similarly, though the therapeutic value of an antiserum may be correlated with some special chemical group in the antigen, it does not follow that it is correlated with each of the antigenic characters which may be demonstrated by agglutination or precipitation. But, with pneumococci, the practical difficulty is to find laboratory data which will enable one to discriminate between important and unimportant antigenic differences; and this difficulty has not yet been overcome.

The significance of serological types of pneumococci therefore demands serious consideration. The subject raises many immunological problems and may be discussed from many aspects which I have not attempted to deal with in this report. All I propose to do is, first to call attention to certain general ideas about antigens and antibodies, and then to apply these considerations to a practical question. Neufeld's standpoint does not appear to hold out prospects of further progress; can one find a wider outlook which will give a more helpful view as to the significance of pneumococcal antigens?

General Ideas about Antigens and Antibodies.

General ideas must be handled with caution. For example, it does not follow that what is known about the antigenic properties of serum or other non-bacterial protein can be applied in every detail to bacterial antigens; and, again, the antigenic characters which are of importance in preparing a serum which will prevent or arrest invasion by parasitic bacteria are not necessarily the same as those which are of predominant influence in the serological classification of bacterial races by means of the agglutination test.

So preliminary ideas may give rise to further questions as to how far principles based on one kind of work (*e.g.*, specific tests for blood serum) are applicable to another branch of immunity (*e.g.*, the production of antibacterial sera). With this understanding, I begin by borrowing freely from the facts, theories, and suggestions which have been put forward by biochemists in their studies of non-bacterial antigens. In what follows, Pick's valuable survey of the subject has been particularly helpful.*

* Kolle and Wassermann's *Handbuch der pathogenen Mikroorganismen*, 2nd Edition, Vol. I, p. 685. 1912.

Chemical Specificity.

One must first try and form some mental picture of what is meant by specificity; and it will be convenient to take the chemical aspect before going on to discuss colloidal conditions.

The balance of opinion is in favour of the view that all substances which act as antigens contain large and highly complex molecules and are of a protein nature, or at least cannot be dissociated from protein.* Pick believes that a further chemical characteristic of an antigen is the possession of an aromatic nucleus. He regards this aromatic complex as the central ring for the grouping of the side-chains, upon the arrangements of which specificity depends, since the aromatic nucleus, as such, cannot possibly account for the enormous number of possible variations which nature affords. This wide range of variation must depend upon differences in the position of different groups, or side-chains, within the molecule, including differences in stereo-chemical configuration.

Remembering that the above conception is to be supplemented, later on, by a consideration of colloidal conditions, and that it by no means implies acceptance of what is known as Ehrlich's theory of immunity, I think it may be regarded as a non-controversial way of forming a starting point in the attempt to arrive at a rough chemical representation of the specific property of an antigen.

The biochemists have provided some interesting data which enable one to fill in the picture with certain details illustrative of antigenic characteristics.

1. *The "dominant antigen."*—If a protein (the serum of an animal) is converted into a nitro-protein by the action of nitric acid, a profound change in antigenic specificity is produced. The original specificity (*i.e.*, the specificity characteristic of the animal species) is lost and is replaced by a new specificity which is common to all nitro-proteins and is peculiar to these compounds. For example, nitrified rabbit serum, when used as an antigen, will produce an antiserum (either in rabbits or in other species of animals) which will precipitate the nitro-protein obtained by nitrifying the protein of any species of animal but will not give a characteristic reaction with normal rabbit serum. Similar changes in specificity are produced by converting serum proteins into iodised proteins or into diazo-proteins; the original serum specificity is lost and a new specificity common to all iodo-proteins, or to all diazo-proteins, is acquired.

A particular chemical factor, therefore, may completely dominate antigenic specificity. Perhaps a factor of this nature is dominant in certain bacteriological species, the members of which appear to be serologically uniform; *e.g.*, it appears to be a general rule that all true vibrios of Asiatic cholera should agglutinate with the same standard serum.

* I think confirmation is needed for the suggestion that protein-free lipoids may act as antigens.

2. *Limitation of specific reaction due to multiple antigenic components.*—Chemical influences of a less drastic nature than the above may alter the original specificity in another way, not by abolishing it, but by adding to the original antigen a new antigenic component which makes the modified antigen less wide in its range of action than the original antigen. To quote an example given by Pick, an antiserum prepared by immunising with diazobenzol-ox-protein will precipitate only diazobenzol-ox-protein, not normal ox-protein and not the protein of man, horse, or dog, which has been turned into the diazobenzol compound. In general terms, when an antigen A (here the serum of a particular species of animal) is linked to a new antigenic character, *b* (here a known chemical compound), the combination A*b* may retain its original specificity (due to A) whilst acquiring a new "constitutive" specificity (due to *b*); thus the antiserum produced by immunising with A*b* may react only to A*b* antigen, not to *b* coupled with an antigen other than A, not to the normal A antigen, and not to A coupled with *c* or *d* or *e*, &c.

One cannot fail to note the resemblance, which may or may not be of intrinsic importance, between these experimentally modified antigens and the idiosyncrasies of certain normal bacterial antigens. To take the readiest example, one may suppose that all pneumococci possess, as such, a common antigenic nucleus (N) and that racial differences are due to the possession, in addition, of special antigenic properties (*b*, *c*, *d*, &c.); it is found, however, in agglutinin and precipitin tests, that N*b* antiserum does not react with N*c* or N*d* antigen, but only with N*b*.

3. *Increased breadth of reaction due to multiple antigenic components.*—It has already been mentioned that a native serum protein (A), the nitro-protein (B) of the same serum, and the diazo-protein (C) of that serum behave as three entirely distinct antigens. But, to quote an example given by Pick, if A is linked to *b* (diazobenzol), and B is linked to *b*, and C is linked to both *b* and *c* (α-naphthol), it is found that the three antisera produced by immunising with (1) A*b*, (2) B*b*, and (3) C*bc* all react equally well with A*b* antigen. Here is an example which is the converse of the last. A, B and C*c* behave as antigens having nothing in common, but the addition to each of the component *b* brings out a very striking inter-relationship between the three antigens thus formed.

This result provides an apparently close parallel to what is very frequently found by bacteriologists. Studies of the serological races of meningococci and of the Flexner group of dysentery bacilli are good examples. Strains are found which behave with apparent inconsistency when different serological criteria are applied to them, being distinct from each other according to some tests and closely akin when other tests are applied. The natural explanation of the resemblances between strains which also differ from each other is suggested by the

artificial antigens last mentioned. Pure A antigen will not react with a pure B serum; but A*b* antigen reacts with B*b* serum.

4. *Concealed antigenic components.*—In the last example taken from experimental biochemistry, the presence of an additional component *b* caused increased breadth of reaction. It does not follow, however, that this result is the invariable rule. In fact, additional details, taken from the protocol of the same experiment, prove that this is not the case. It is shown that the antiserum A*b* forms no precipitate with the antigen D*b* (D being the iodised form of ox-protein); nor does it form any precipitate with B*b* antigen; and it gives only a slight reaction with C*bc* antigen. Thus *b* antigen and *b* antibody may fail to detect each other, evidently because the relations of *b* to the other constituents of antigen or antibody are highly complex; they are by no means a matter of simple juxtaposition, but depend upon a variety of differences in chemical structure; and these differences may be of such a nature as to interfere with the reaction.

Here, perhaps, may be found the explanation of one of the puzzles which sometimes arises in trying to apply the doctrine of multiple antigenic components as an explanation of the relationships between different serological races of a particular bacterial species. It is postulated that a particular race of bacteria has *b* as one of its antigenic components; in some reactions, as exemplified above, there appears to be clear evidence of this, but, when other diagnostic tests are employed under apparently identical conditions, the expected response is not forthcoming. The explanation of the negative result may be that the postulate is right, but the presence of *b* is sometimes masked.

Chemical Instability.

It has been shown experimentally that antigens are highly susceptible to modification by physical or chemical influences such as heat, cold, formaldehyde, chloroform, dilute alkali, &c., *i.e.*, by influences which are not sufficiently potent to produce profound changes in protein structure. This emergence of new antigenic characters, with partial or complete disappearance of some of the old characters, is naturally to be explained by chemical instability. The antigenic substance originally contained groupings which were arranged in a particular way, but, under the physical or chemical influence in question, some of the groups, or the arrangement of the groups, became altered. The new groups, or arrangements, did not exist, preformed, in the original antigen; all one has to postulate is that the original groups were readily susceptible to modification.

This view, which is accepted without question in relation to these artificially modified antigens, is regarded by many biochemists as equally applicable to natural processes of immunisation. The substances capable of acting as antigens are labile,

and, in the course of metabolism, particularly in the living body, pass through a large variety of changes, including chemical grouping and re-grouping, which are dependent on the conditions of metabolism. New groups of antigenic value (*i.e.*, new antigens or new antigenic components) may emerge at any stage of these processes, just as some of the old groups (*i.e.*, the original antigen) may be lost.

This conception of chemical instability in the antigen, which I think must be accepted as reasonable, has an important bearing on one's ideas of "antigenic components." In one important respect it does not supersede the idea, current amongst bacteriologists, of multiple antigenic components existing side by side. In the preceding paragraphs, examples have been given which illustrate the elements of truth in that view. But, in another and perhaps more important respect, the idea of the evolution and devolution of antigenic properties as a normal event of metabolism is incompatible with a strict interpretation of the "mosaic pattern" hypothesis, according to which every demonstrable antigenic function is referable to a special chemical group, preformed in the original molecule. The difference between the two views may be expressed briefly. According to the former, an antigenic function is referable to a chemical group *b*, which may be either (1) pre-existent in the original molecule and in an active state therein, or (2) pre-existent in the original molecule but in a masked condition and only able to manifest its activity if subsequent changes remove inhibitory conditions, or (3) non-existent in the original molecule and newly created in the course of subsequent changes. According to the latter view, *b* must be either (1) or (2).

Possible examples of (3) would be :—(a) production of agglutinins by immunisation with an inagglutinable strain of bacteria, or (b) production of antitoxic sera by immunisation with bacteria which, in the intact condition, are non-toxic, or (c) production of sera which are antibacterial *in vivo* by immunisation with bacteria towards which the sera are not antibacterial *in vitro*, or (d) modification of antigenic character by tryptic digestion.

But the alternative view, which would eliminate (3), cannot be disregarded. It raises wider questions, which will now be considered more fully, about the value of Durham's hypothesis as an explanation of immunity.

The "Mosaic Pattern" Theory.

According to this doctrine, antigenic characters consist of certain preformed chemical groups which are present in the protein molecules. There may be several of these groups. They are often imagined as forming a mosaic pattern, the complexity of which corresponds to the range or complexity of the antigen. In the production of an immune serum, each unit of the antigen mosaic gives rise to a corresponding or counterpart group in

the serum. These counterparts, attached to certain elements in the serum, form the complex known as antibody. The antigen-antibody reaction consists in the union, subject to favourable colloidal conditions, of preformed antigen groups with their preformed counterparts or affinities which are present in the antibody. This is a development of the well-known " lock and key " conception of Emil Fischer, which has proved of great value in many respects.

Difficulties arise at once in the application of this conception to known facts. Normal sera react with a very large variety of substances in a way which cannot be distinguished from an antigen-antibody reaction. It must therefore be postulated that these non-specific reactions, also, are due to structural groups, each " fitting " exactly with a corresponding group in any antigen with which it happens to react. So the mosaic pattern of an immune serum must be sufficiently complex to include its " mosaic " as a normal serum along with its special " mosaic " which it has acquired by immunisation.

There is a further complication in the pattern. In the production of antibodies, non-specific factors frequently play an important part. For example, the agglutinin produced by immunising a rabbit with a particular bacillus may be due not merely to the specific antigenic group but to the complex of specific and non-specific elements contained in the inoculum. The reason for this supposition is the observation that a rise in titre for the same strain is often obtained by subsequent inoculation with a bacillus of quite different species. Or, again, it has been alleged that protection against a particular bacterium may sometimes be obtained by vaccination with an entirely different virus, or, possibly, by using a vaccine which is not prepared from any bacterial protein. In general, there are many indications that great importance should be attached to this " non-specific " acquired immunity.*

According to the " mosaic " conception, all the above facts and difficulties are to be accounted for by postulating that a bacterial antigen consists of an indefinitely large number of parts, *a*, *b*, *c*, *d*, &c., and that the specificity resides in the complex as a whole, though certain of the components also occur in the antigens of other bacterial species or even in non-bacterial protein. This overlapping of *a* or *b*, &c., and the overlapping of the corresponding constituents in the antibodies would account for non-specific influences. In other words, the non-specific element is to be explained by an indefinite multiplication of the units which are supposed to explain specific reactions.

In discussing the value of the postulate which I have outlined in the preceding paragraphs, one must first note that it is biological rather than biochemical, in the sense that it is derived

* Here I am assuming, provisionally, that the antigen-antibody conception suffices to explain immunity. In a later section (pp. 59–63) I suggest that this assumption may not always be valid.

from immunological data which cannot be expressed in precise terms of chemistry or physics. This is necessarily the case in dealing with the extremely complex material which confronts the bacteriologist, where antigens cannot be identified, even partially, with known chemical constituents, and where the results of antigen-antibody reactions are simply immunological facts for which a precise chemical or chemico-physical explanation is not available. Perhaps it may be said that the constituents of the " mosaic pattern " are really no more than postulated biological factors. In defence of this attitude, it may be pointed out that progress would be impossible for the immunologist if he could not advance beyond the narrower and more precise sphere of the biochemist.

It is, of course, always very desirable to find biochemical data which support the necessarily vaguer conceptions of the immunologist. It is therefore satisfactory to observe that a " mosaic " conception fits some of the facts established by the biochemists, *e.g.*, selective affinity between particular groups in the antigen and particular groups in the antibody. I refer, in particular, to the biochemical studies already mentioned where antigens have been modified by the introduction of known chemical groups and comparison has been made between the antibodies produced by the original and by the modified antigen.

But, taken as a general explanation of immunity reactions, the " mosaic " theory travels far beyond the confirmatory data of the biochemist. This might not be objectionable, if it could be shown that the postulate of an indefinitely large number of biological units was not incompatible with any well-founded deductions from biochemical facts. It is this last consideration which raises awkward questions.

Many substances capable of acting as antigens or antibodies have an indefinitely large number of combining capacities. That is agreed. On the " mosaic " theory, these combining capacities are units in the mosaic which pick each other out, leaving the rest of the mosaic as it was before. This would only be possible under one condition, viz., that there was a different and independent protein vehicle for each different unit.

Is this last suggestion to be taken seriously? It may seem an easy way of providing *ad hoc* explanations for particular results. In agglutination work, for example, it may be postulated that the antigens of a certain bacterial strain consist of different proteins *a*, *b*, and *c*, which are chemically independent of each other, though perhaps loosely united as a colloidal complex; in this strain, *a* may be in excess of *b* and *c*, whilst, in another strain, *b* may be in excess and *a* may be absent, and so forth; and corresponding differences (*i.e.*, presence, in greater or less amount, of different specific proteins), would be postulated in the antibodies evoked by these antigenic groups. The objection to the hypothesis is that, if it is to be taken as of general application, the principle which applies to one particular set of

observations on immunity reactions must apply to all; and that leads to the assumption that any given bacterium or serum must be provided with an indefinitely large number of different proteins, an assumption which appears to me to be arbitrary and improbable.

On the other hand, unless some such hypothesis as this is to be accepted, difficulties arise. For, if these different units of the mosaic are to be regarded as groups or side-chains linked to the same molecule, or aggregate of molecules, the biochemist has the right to point out that the idea of picking out bits from a mosaic and leaving the rest intact is quite incompatible with what is known about the structure of organic chemical compounds. The slightest alteration in a chemical group may bring about a profound modification in the molecule as a whole; and one cannot suppose that the molecule could be split up into an indefinite number of integral component parts, each functioning as antigen or antibody. For example, the picking out of agglutinins from an antibody, by successive treatment with different antigens, cannot, on this view, be accepted as literally true, but only as a conveniently short expression for the production of complex and imperfectly understood changes in the antiserum concerned.*

It is possible, however, that I may be debating this question with too much solemnity. Some supporters of the "mosaic" theory may refuse to be forced into the dilemma of deciding whether every unit in the pattern is to be regarded as an independent protein or as merely a side-chain. They may say that in some cases, as in the example from agglutination work which I have quoted, the postulate of three independent proteins is quite reasonable and affords a good explanation of the facts; but it does not follow that every different immunity reaction means the presence of a corresponding, and independent, protein in the antigen, together with a corresponding protein in the antibody; the same protein, by virtue of its complex chemical structure and its variety of side-chains, may exercise many different functions; hence the admittedly great diversity of immunity reactions by no means implies an equally great diversity of independent proteins. This seems to me to be quite a plausible compromise, if it is to be interpreted as meaning that the "mosaic" idea must not be pushed too far—in other words, that it must not be taken too seriously.

A further Antigenic Requirement.

Protein character and colloidal condition are not the only two requisites for an antigen; it must also be in such a condition

* In a report on meningococci I have discussed in detail the difficulties which may arise from an attempt to subdivide a bacterial species by selective absorption of agglutinins. (*Reports to the Local Government Board on Public Health and Medical Subjects.* New Series, No. 114. 1917.)

as to stimulate the production of antibodies. This third factor is very difficult to define. It is usually described by saying that the antigen must be "foreign" to the animal body in which an immune response is to be excited; the difficulty is to give precise expression to what is vaguely described by saying that the "foreign" protein must be different from the protein constituents of the animal body with which it reacts. One may say that the antigen, being introduced parenterally, must cause some disturbance in the general metabolism of the body, and thereby stimulate the production of some new mechanism, possibly an enzyme, for dealing with the altered conditions. But this disturbance need not be great. No marked changes in protein metabolism need result from the introduction of foreign protein; and a single small dose of antigen may give rise to a plentiful supply of antibody.

It is obvious that this difference between the chemical or chemico-physical structure of an antigen and the proteins of the animal to be immunised must be retained long enough for the antigen to act as a stimulus. Hence good antigens possess molecules of large size and are not readily broken up. In accordance with these observations, it is found that antigenic properties are readily lost when the antigen is exposed to the action of proteolytic ferments.

It is of interest to note, however, that some antigenic capacities may, in a modified form, survive this treatment. For example, Obermeyer and Pick exposed bovine serum to prolonged tryptic digestion and found that the digest produced an immune serum which formed a precipitate with the digest but failed, or almost failed, to react with the undigested material. But the specificity characteristic of the animal species was retained completely; their immune serum gave no reaction with the tryptic digest of horse serum.

This example of antigenic change resulting from a process analogous to metabolism in the body should be borne in mind as a possible explanation of antisera which are efficient *in vivo* but do not react with antigen *in vitro*.

Influence of Physical Conditions on Specificity.

The most important of these conditions is the fact that the reactions of antigens are inseparably associated with their colloidal state. Hence it follows that reactions between antigens and antibodies are conditioned by the laws which govern the interaction of colloids.

A few of the more important consequences which may follow from this circumstance are worth considering.

1. *Quantitative Relationships.*—One has to remember that, in addition to qualitative conditions of a chemical or physico-chemical nature, the quantitative relationships of the interacting substances are of special importance in colloidal reactions. In

immunity work, therefore, care has to be exercised in establishing the quantities of the reagents which will secure an optimum reaction.

The importance of this point is well illustrated by " inhibition zone " phenomena.

2. *Differences in the Mechanism of Reaction.*—These may be largely influenced by colloidal conditions. According to Landsteiner, there are two general types of immunity reactions. In the first, as exemplified by agglutination, precipitation, and the union of toxin with antitoxin, there is the simple union of two colloids. In the second, as exemplified by cytolysis, colloids (or a complex colloidal mixture) bring about a destruction of lipoid-protein combinations, or of cell-membranes which contain lipoidal constituents, the result being the release of soluble cellular contents. Another important fact is that the presence of a third colloid, such as normal serum or even an inorganic colloid, may profoundly influence the behaviour of antigen and antibody.

Such circumstances as these show that the reactions of antigens and antibodies cannot be expressed in terms of merely chemical combination.

3. *Conception of a " Pure " Antigen.*—From chemical considerations alone, it has appeared necessary to postulate that the special chemical groupings which characterise an antigen can only exercise their functions when united to a protein molecule, and that, therefore, it is impossible to effect a chemical separation of the purely specific antigenic components from the rest of the molecule, or to regard the protein as merely an unessential attachment to the antigen. This difficulty, as regards the individuality of an antigen in its chemical aspects, becomes still greater when the essential colloidal attributes of an antigen are considered. Whilst not disregarding the importance of chemical constitution, it has to be remembered that the participants in antigen–antibody reactions are large colloidal complexes and are not substances possessing chemical individuality in the ordinary sense in which this term would be understood by the organic chemist.

Hence a " pure " antigen is an abstract conception which is very different from the concrete reality.

4. *End-Results depend on a Sequence of Reactions.*—According to the biochemical view, the specific activity of an antigen does not depend merely on a particular grouping of atoms within the molecule or on one special physico-chemical constant; specificity is the resultant of a long series of different phenomena which follow one another in causal sequence, just as the unlocking of one door gives access to a second, and the unlocking of the second to a third. Immunity is particularly concerned with a special class of these " linked " reactions, viz., with those processes where an intermediate reaction is required to provide the energy necessary for a further reaction, for example, when *a* must first

unite with *c* in order to acquire the energy for union with *b*. Pick's suggestion is that, in antigen-antibody reactions, the colloidal reaction is intermediary and provides the energy necessary for the subsequent reaction which involves changes in chemical structure.

This view, it will be observed, demands some expansion of ideas about specific reactions in the animal body. In some cases, a simple explanation may suffice. The reaction may commence with a specific union between antibody and bacterial antigen; and then the bodily mechanism may step in and complete the antibacterial process in a non-specific manner. But, in other cases, the process may be much more complicated, and it may be impossible to draw a hard-and-fast line between a specific and a non-specific part of the reaction. For example, the antibody, in the form of an immune serum used for conferring passive immunity, may react first with some constituent of the animal body, as a preliminary to acquirement of energy for specific action on the bacterial antigen; *i.e.*, the specificity of the reaction may be due to the combined influences of antibody and the animal's natural mechanism of resistance, not to the former alone.

5. *Chemico-Physical Equilibrium in Relation to the Conception of Antibodies.*—" Physiological equilibrium " and " the disturbance of this equilibrium in disease " are phrases which are readily understood; but the relation of " equilibrium " to antibodies requires some explanation. Perhaps this subject may be introduced by describing it as an attempt to give more accurate scientific expression to what, according to the Ehrlich school, would be termed a " habit." I refer to the familiar doctrine that the reason why an animal keeps on turning out amboceptor, long after the antigen which provided the original stimulus has disappeared, is that the animal's cells have acquired the " habit " of doing so. If the animal's mechanism were simply a complicated problem in mechanics, one might substitute " resultant " for " habit "; *i.e.*, the normal play of interacting forces has a particular resultant force *a* as its outcome; but these interacting forces are so numerous and complicated that the slight jar caused by a new force *b* (the antigen) dislocates the whole mechanism; hence it is not a case of finding the resultant between *a* and *b*; the new resultant *c* is mainly due to a rearrangement of all the forces which previously gave resultant *a*; and, therefore, *c* may persist after the relatively insignificant force *b* has disappeared. But, of course, " resultant," as the term is used in elementary physics, is not adequate to explain vital phenomena.

Vital processes may be regarded, in one aspect, as interactions between the innumerable colloidal constituents of the humoral and cellular elements of the body. These interactions, at any given time, tend to find an equilibrium which is determined by the quantitative as well as by the qualitative conditions of the interacting substances, and represent the potential properties

of the living plasma and cells of the normal animal. As the animal's individuality is the resultant of all the bodily forces at work, this equilibrium may be regarded as an expression of that individuality. The equilibrium is disturbed when a new factor is introduced, such as the presence of a foreign protein; and the disturbance may result in the temporary or permanent establishment of a new equilibrium. The new equilibrium makes a difference in the animal's vital reactions, and this difference may be particularly noticeable on re-introduction of the foreign element which caused the original disturbance. The new equilibrium, it is suggested, may be regarded as the real explanation of a property (acquired immunity) which is more usually attributed to a newly formed special substance (antibody); just as natural immunity may be explained by the old equilibrium rather than by a supply of natural antibodies.

This view, though not sufficiently tangible to be regarded as amounting to a theory of immunity, may be useful in two respects. (1) It is certainly desirable to get rid of the word "habit," which is meaningless when offered in explanation of biological data. On the other hand, the conception of varying conditions of chemico-physical equilibrium is based on experimental facts; and, though these experiments do not reproduce the much more complex interactions which take place in the living body, there can be no doubt that questions of "equilibrium" are of high importance *in vivo*. (2) In the desire for simplification, there is a natural tendency to explain a result as due to the operation of a single force, *e.g.*, to the action of a particular substance termed an antibody. But the real explanation may be much more complex, owing to the large variety of forces or circumstances making up the complete causal nexus of events to which the result is really attributable. What has been said above about colloidal equilibrium may be taken as an expression of this more complex conception.

This idea of equilibrium may also be correlated with another way of attempting to explain a well recognised difficulty. In the animal body there are many substances (ferments being the commonest example) which are known to possess active properties but do not exert their activity upon the living material of the body. By way of explanation, it has been found necessary to suppose that in the living animal there are various inhibitory or antagonistic forces, acting as "buffers" or as "antienzymes" or in other ways, and that these prevent the occurrence *in vivo* of reactions which readily take place *in vitro*. This idea that "anti" substances are part of the balanced mechanism of the normal animal has found its way into discussions of some of the obscure problems of immunity. I take the following example, which is an endeavour to find some common ground for the phenomena of natural and acquired immunity. It is based on the hypothesis that all animals possess, in greater or less degree, powers of resistance against any particular bacterium, and that the differences between the highly susceptible, the moderately

resistant, and the naturally immune animal are only differences in degree. The explanation, then, would be that in the naturally immune animal the interchange of bodily activities, termed metabolism, is unfavourable to the multiplication of the bacterium in question because these activities are balanced in such a way that none of the interacting colloids are free to inhibit antibacterial action. The serum of such an animal need not be bactericidal *in vitro* and need not be protective, in ordinary doses, because the interacting forces between which the equilibrium exists are only active in the living body and are not represented in the serum. In the susceptible and normal animal, it is to be supposed that, when the bacterium is introduced, there is some " buffer action " which tends to inhibit the animal's natural antibacterial forces; the result of the infection will depend on the degree of this intervention, being in favour of the bacteria if the interference is great and in favour of the animal if it is too small to be permanently effective. When an animal, originally susceptible, has acquired active immunity, it is to be assumed that immunisation has produced a new equilibrium, which has been adjusted in accordance with the influence of the immunising antigen and has eliminated the " buffer action " for that particular bacterial type. This condition of active immunity need not be accompanied by demonstrable antibodies, and, for the same reasons which applied to the naturally immune animal, the serum may not be bactericidal *in vitro* and may not confer passive immunity.

It appears to me that attempts, such as the above, to apply ideas of a balanced mechanism to problems of immunity are not substantial enough to carry conviction. They are obviously not intended as an alternative explanation in cases where the immunological data are fairly simple. For example, there is a recognised association between resistance to diphtheria and the presence of antitoxin in the serum; this is a concrete fact which goes a long way towards explaining immunity against that disease, though it may not be the complete explanation. And there are many other well known instances where susceptibility or resistance to infection is correlated with the presence or absence of demonstrable antibodies. In such cases, theories about equilibrium would not serve any useful purpose. But in other and more obscure cases, where immunity cannot be shown to be due to an appropriate antibody and where no substantial or convincing explanation is forthcoming, speculation is desirable and hypotheses about equilibrium are one of the subjects which is entitled to some consideration. Such matters may at least serve to raise the question, which I discuss in the next section, whether there is not a danger of placing too much reliance on individual antigens and antibodies as explanatory of immunity.

Possible Limitations of the Antigen-antibody Conception.

It is probable that, in addition to the known types of antibodies, such as precipitins, agglutinins, antitoxins, bacterio-

tropins, and so forth, many other kinds of antibodies remain to be discovered. This does not necessarily imply the existence of an indefinitely large number of separate substances; some, at least, of the antibodies which differ from each other may be thought to be merely different properties of the same substance.

Still, though the antigen-antibody conception of immunity (as being concerned with the union of two substances possessing chemical affinities) will doubtless lead to further discoveries of importance, there are reasons, emerging from the biochemical aspects of immunity, which indicate, to my mind, that demonstrations of antigens and antibodies are only one aspect of immunity phenomena. Such phenomena are essentially interactions between protein colloids, not between parcels of antigens and parcels of antibodies. These colloids, it is true, may behave in some respects as antigens and antibodies; but that is only part of their behaviour, perhaps not always the most important part, and certainly not the sum and substance of their chemico-physical interactions. Therefore it seems desirable to guard against making the assumption, perhaps unconsciously, that the discovery of fresh reactions between antigen and antibody is the only way, within the present limitations of knowledge, in which further light can be thrown on immunity. Immunity problems are not limited to the discovery of the right antigen for the right antibody.

In the first place, it is customary and proper to introduce a discussion on immunity with the admission that very little is known about it. All the main facts depend upon the vitality of the animal body, the mechanism of which is too complicated to be explained by known methods of scientific analysis. Immediately after death, and before gross chemical or physical changes have occurred, the body loses its capacity to resist bacterial invasion, the reason evidently being that this capacity is associated with extremely labile activities which become inert as soon as the supply of oxygen and other essentials for vitality is cut off. Vital substances cannot be preserved in their active state for examination and analysis in the laboratory. Observations may be made on the properties of fresh blood, plasma, serum, or leucocytes; but the most that can be claimed for such experiments is that they may represent a faint and very imperfect reflection of vital phenomena; it has to be admitted that they give very little information about what actually takes place in the animal body, though of course, they may afford evidence that the animal body has acquired new vital properties. The facts termed vital resistance comprise a big and almost unexplored territory, which may serve as a reminder that it is unsafe to base an imposing structure of theory on conclusions which travel beyond the facts. For this reason it is doubtful whether the search for antigens and antibodies which are at present unknown is all that is needed to provide a more solid basis for this structure.

Another limitation to the antigen conception arises from the fact that one requirement of an antigen is that, in order to stimulate the production of an antibody, it must possess something (*see* p. 54) which is "foreign" to the immunised animal. Foreign protein stimulates the animal body to produce certain antibodies such as precipitins, agglutinins, bacteriotropins, or antitoxins; and the methods of production, if not the actual substances produced, are probably very much the same in each case, the differences being mainly attributable to the antigens. But the reactions of the animal body towards dead, or non-cellular, foreign protein, though complicated enough, are much less complex than the reactions which occur during the temporary symbiosis between living parasitic bacteria and their animal host. The living bacterial cell is much more than an antigen ready for the reception of antibodies; and its postulated "antigen" is not fixed but is always undergoing metabolic changes, which depend upon all the constituents of the bacterial cell and upon its interactions with the tissues and fluids of the animal host. Moreover, though dead bacteria are foreign protein, a bacterial parasite, when thriving within the animal body, is not behaving like foreign protein; so it cannot be assumed that necessarily the best way to inhibit its growth is to discover an antibody to its dead and disintegrating remnants, or that this is nature's way of acquiring immunity.

Three of the commonest and most important facts about natural immunity are :—(1) resistance to saprophytes; (2) resistance, on the part of a susceptible animal, to a small dose of parasitic bacteria; (3) resistance of an animal belonging to a naturally immune species. It cannot be shown that serological antibodies to bacterial antigens are the explanation of any of these three kinds of immunity.

In immunity acquired by vaccination there are two facts to note :—(*a*) increased resistance, and (*b*) generally, though not invariably, the appearance of a serological antibody to the injected protein. The second fact is undoubtedly associated with the first, but can it be assumed that (*b*) is the complete explanation of (*a*)? It is hardly reasonable to think that there is a sharp gulf between acquired immunity and types of natural immunity such as (1), (2) and (3); I would prefer to assume that in the former, as in the latter, factors other than antibodies are of importance. Similar considerations may apply to passive immunity. Many bacterial infections, notably anthrax, fowl cholera, and swine erysipelas possess two features in common; their specific immune sera are not bactericidal *in vitro* and attempts to explain the immunity by demonstrating specific antibodies have failed to gain general acceptance. Quite possibly the reason may be that the enhanced resistance of the immunised animal is not due, or not wholly due, to the direct or indirect action of an antibody on bacterial antigen.

It has been suggested above that, in some cases, there may be no demonstrable antigen-antibody reaction in association with

resistance to infection and that, in other cases, such a reaction may be demonstrable but may be insufficient to account for immunity. At this point it is natural to raise questions as to the importance of complement and phagocytosis, either as acting independently of an antigen-antibody reaction or as the agents for carrying on the work initiated by such a reaction.

Questions about complement and phagocytosis form part of a larger problem, the mechanism of vital resistance, which, though it must be viewed from many different aspects, cannot safely be split up into smaller, water-tight compartments. There is no question of choosing between a " cellular " and a " humoral " theory, because the cells and the plasma are equally essential parts of the mechanism, and the condition and contents of cells and plasma are interdependent. Nor is the importance of the cells confined to the phagocytes. Practically all the tissue cells which take any part in the metabolism of the body must be regarded as concerned with immunity in greater or less degree. Particular mention may be made of the endothelial cells, which are present in every part of the body and, apart from their work as phagocytes, act as selective filters between the blood and lymph streams and the tissues. It is therefore dangerous to focus attention exclusively upon one type of cell, *e.g.*, to conclude from *in vitro* experiments upon leucocytes that the animal's protective mechanism in a particular instance is, or is not, phagocytosis of sensitised bacteria.

It has long been known that circulating plasma and serous exudates contain substances, other than antibodies, which play a part in immunity, though the precise nature of these substances has never been defined. In the older literature, substances of this sort are often called complement or alexin, terms which, when used with reference to the living body, cannot be accepted as meaning more than postulated factors of unknown nature. But to assume that these factors, or some of them, are identical with the properties of fresh guinea-pig serum, which is the ordinary laboratory sense in which the term complement is used, would not be justifiable. For example, the absence of bacteriolysis in a test-tube experiment, where fresh guinea-pig serum is one of the reagents, would not justify any inference as to the protective mechanism in the body of an animal infected with the bacteria under investigation. It is not at all clear that this laboratory reagent is, or contains, any special substance which is a common constituent of all animal bodies. I have discussed the question at length in relation to the Wassermann test for syphilis.*

It seems to me, therefore, that antigens and antibodies, even when supplemented with postulated functions of complement and phagocytosis, are not a sufficiently large stock-in-trade of ideas to explain immunity.

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* *Ministry of Health. Reports on Public Health and Medical Subjects.* No. 1. 1920.

The difficulty may be expressed in another way by raising the question—How far does the mechanism of acquired immunity resemble natural immunity? Very little reflection is needed to realise that practically nothing definite is known about natural immunity. Then what part is played by the new factor which is operative in acquired immunity? Is it simply the removal of some obstacle (or the addition of some adjuvant) whereby the forces of natural immunity become effective? Or is it a new and independent mechanism? Perhaps neither alternative is satisfactory; but it seems probable that the former alternative contains the larger element of truth, and that, therefore, the mechanism of acquired immunity largely depends upon factors which are unknown.

Discussion of Pneumococcal Antigens and Antibodies.

The above discussion of general biochemical conceptions of specificity, though not as a rule based on work with bacterial protein, provides some suggestions which may be utilised in discussing pneumococcal antigens and antibodies.

Antigenic Varieties or Variants.

It may be assumed that, in reality, the pneumococcal antigen is a very complicated affair, and that its specificity depends on the mode of union of particular chemical groups with a protein nucleus, on the particular colloidal condition and environment of its composite molecules, on its particular capacities for change in a given sequence of interactions, and so forth. All this may be called, for short, the "antigenic complex."

As they form a moderately well-defined species, with bile solubility as one of their characteristics, it is reasonable to suppose that all pneumococci possess many properties in common, as regards chemical structure and composition. But these common properties are not demonstrable as a common antigen. Antigenically, pneumococci exhibit a perplexing number of varieties. Perhaps these two circumstances may be brought into proper association by describing the antigenic varieties as variants of the common pneumococcal structure.

The variants differ from each other in respects which may perhaps be small, such as differences in position or structure of a particular chemical group attached to the molecules constituting the antigenic complex. But it is known that a slight chemical change may suffice to bring about a profound modification of antigenic properties. The special feature of the pneumococcal variants is that each imposes on the complex a marked limitation of specificity, as is demonstrated by agglutination and precipitation tests. These circumstances may be illustrated by the example, given on p. 49, of " limitation of specific reaction due to multiple antigenic components." It is not necessary to assume that the differences of the variants are due to more profound

structural differences, such as are termed, on p. 48, differences in the "dominant antigen." I am not suggesting that this latter hypothesis can be excluded, but merely note that it does not appear to be necessary.

With some bacterial species, different races are found to possess certain serological attributes in common, though differing in other serological respects. This overlapping of the serological reactions is naturally explained, as illustrated on p. 49, by the postulate of multiple antigenic components. It is also probable that, in strains belonging to such species as these, common antigenic constituents may sometimes be present, though failing to respond to the appropriate antibody, as in the example (p. 50) of concealed antigenic constituents. But when, as is the case with different races of pneumococci, serological interrelationship is not demonstrable to any conspicuous extent, there is less support for the idea that such "masked" antigenic constituents are a common property of the variants. Still, this view is theoretically possible. Hence the justification for the working hypothesis to which some investigators still adhere, that serological reactions will ultimately be found which will unmask these concealed constituents and demonstrate serological properties common to all pneumococci. This possibility may be taken as a reason for saying that the present method of serological classification need not be taken as final, though the way of finding a better method may not be clear.

Dealing with the position as it now stands, there appears to be an indefinitely large number of variants, and therefore it may be impossible to make an exhaustive serological classification by means of agglutinins. The attempt may be made to classify as many strains as possible into "races" and thereby to reduce the unclassifiable strains to a minimum; but one doubts if it would be profitable to follow up this line of enquiry very far.

Hence many investigators, though not all (*see* pp. 14–18 of my preceding report), have abandoned the attempt to make a polyvalent or omnivalent serum which would contain the special antibody for each variant. They think it more useful to concentrate attention on the relatively small number of varieties which comprise the majority of the strains isolated from cases of lobar pneumonia.

Chemical and Colloidal Instability.

The questions of "chemical instability" discussed above (pp. 50–51) lead one to consider whether the different antigenic variants of pneumococci are really "fixed" and sharply or permanently distinguishable, one from another.

Some laboratory evidence of antigenic relationship has been demonstrated between different serological variants; but, as I have already remarked, such resemblances appear to be of minor character, the more striking feature being that the variants

are antigenically independent of each other. It also appears that these differences are generally stable, under ordinary laboratory conditions. Owing to the particular structure of the "antigenic complex" in each strain, common properties seem unable to find antigenic expression.

But are any or all of these antigenic types equally "fixed" in nature, and incapable of modification in the animal body? Since the biochemists have shown that a relatively slight chemical or physical influence may profoundly alter antigenic characters, it would seem probable that natural processes may accomplish similar results. As the animal body is very commonly capable of annihilating the whole antigenic complex of the pneumococcus, it may, perhaps, be capable, either directly or indirectly, of producing the less drastic changes required for transforming one antigenic variant into another. At least, the balance of probability seems to be in favour of this view, although there is an obvious difference between modification and destruction. Attempts to decide the matter experimentally are confronted with the familiar difficulty of discriminating between a modification and a mixture. As the pneumococcus is ubiquitous, the demonstration that one variant has taken the place of another in the body of an animal does not prove that the latter has been modified into the former; the result may have been due to selective action on a mixture.

Perhaps some antigenic complexes are, in nature, more easily modified (*i.e.*, converted into different antigenic complexes) than others, but there does not seem to be any definite evidence that this is the case with different strains of pneumococci. There is some suggestion, however, that certain influences, *e.g.*, those which initiate disease, may tend to modify types in particular directions, and that other influences, such as are prevalent in recovery, may have a diverse tendency. This may be the reason why certain types, such as I and II in some countries, are relatively frequent in lobar pneumonia and usually disappear in convalescence. Of course, this view that types mutate in the course of infection is only hypothetical; but the opposite view, that types may be annihilated but cannot be modified, is at least equally problematical. It seems to me to be begging the question when one designates as "fixed" types those which are found to be relatively more prevalent in pneumonia.

In relation to the "mosaic pattern" conception of antigens, discussed on pp. 51–54, it will be remembered (*see* pp. 16–18 of my preceding report) that French investigators who have been working recently on pneumococci apparently accept this theory as a satisfactory explanation of antigenic complexities, and make it the basis of an elaborate and ingenious classification of pneumococci by means of agglutination tests conducted according to a special technique. And a similar explanation is offered for antipneumococcal sera; they are supposed to contain antibodies corresponding to a large variety of constituents present in the

antigenic "mosaic." These are matters of importance, particularly in view of the efforts which are now being made to establish uniformity in the typing of pneumococci and in the standardisation of pneumococcal antisera.

The immediate difficulty is that it does not seem possible to reconcile this theory with the work of other observers, the special feature of which is the absence of any demonstrable "mosaic" in the pneumococcal antigen; in their experience, agglutination, precipitation and protection tests fail to show substantial affinity between antibodies produced by one type and any hypothetical antigenic components of another type. It is theoretically possible, as I suggested on p. 49, that all pneumococci possess a common antigenic nucleus, N, which is always linked with special antigens *a*, *b*, or *c*, &c., in such a way that the special antigen controls the reaction, *e.g.*, N*b* will only react with N*b* antibody, not with N*a* or N*c*. But even this proposal, which postulates an undemonstrable N, would not serve to explain the differences between the French school and other observers.

For my own part, I think that the mosaic conception cannot be applied in a literal sense or in amplified detail to pneumococci. If, however, it implies no more than an array of biological factors, many of which are unknown, it must be readily conceded that research which may lead to fresh discoveries of such factors is eminently desirable.

On p. 55 it is suggested that some substances may lose their "foreign" characteristic (*i.e.*, their capacity for acting as antigens) more readily than others when introduced into the animal body. If the value of a good therapeutic serum for parasitic bacteria depends on the presence of a special antibody, distinct from agglutinins and precipitins, it is theoretically possible that the difficulty of producing such sera in the case of pneumococci may be associated with a high degree of instability in the antigen required to produce this antibody.

Coming to the question of physical influences and, in particular, to the significance of the fact that the interacting substances are colloids, fresh difficulties arise, which, it would appear, tend to confuse rather than to clarify the problem. Unfortunately, these difficulties cannot be evaded. Owing to the importance of colloidal conditions, it has become necessary to abandon the conception of immunity as a purely chemical affair of receptors and side-chains. These colloidal conditions cannot be treated merely as conveniences which serve as a good excuse for getting rid of some of the encumbrances of the side-chain theory; they are awkward and puzzling facts of an extremely obscure nature. They imply that quantitative conditions play an important part in immunity reactions, and that these reactions, owing to the complexity of varying colloidal influences, do not follow ordinary principles of chemical combination and dissociation. Hence the idea of a "pure" pneumococcal antigen, which presents difficulties from the standpoint of chemical structure alone, seems

to become more and more of an abstraction when one remembers that the real substance possessing antigenic characters is a colloidal complex, that it is intimately dependent on its relations to other colloids, and that its fate depends not on a single reaction but on a sequence of events, wherein the resultant of one reaction determines the reaction which follows.

There is at least one definite conclusion to be derived from the thought of all these complexities; the antigenic properties of pneumococci which have been demonstrated by Neufeld and his successors cannot lead to final conclusions, because they are obviously very far from being the sum and substance of the interactions which take place between pneumococci and the living body.

On pp. 57–59 I discussed the idea that chemico-physical equilibrium is a factor which may considerably modify current notions about antibodies. This attempt to correlate the properties of antibodies with what may be termed biological equilibrium seems to imply that, though the importance of chemical influences cannot be ignored, the individuality of an antibody is to be regarded as the expression of a particular balance of colloidal forces rather than the attribute of one particular chemical structure.

Is this line of thought worth considering in relation to pneumococcal immunity? I concede at once that it does not directly point the way to new avenues for research; but it may serve as a useful corrective against ideas which tend to the opposite extreme of explaining results as due to the interplay of isolated factors, irrespective of their environment. The conception of pneumococcal antibodies as hard and fast substances, each with an individuality of its own, cannot be regarded as established irrefutably and may be incompatible with colloidal conditions of metabolism.

These chemico-physical considerations suggest that the more popular conceptions about immunity are inadequate, but do not provide as an alternative any very helpful working hypothesis, because colloidal reactions in the living body are too complex for analysis. Still, it may be useful to be confronted with these difficulties, if only to prevent one's ideas from running in too narrow a groove.

Therapeutic Value of Pneumococcal Antibodies.

On pp. 59–63 I discussed possible limitations of the antigen-antibody conception. It remains to consider whether these suggestions are applicable to pneumococci.

To begin with what is known about the properties of pneumococcal antibodies the existence of which has been demonstrated experimentally, the results of protection tests on mice have made it obvious that certain specific substances prevent the corresponding variety of pneumococcus from gaining an initial foothold in the animal body. Here two assumptions might be made.

(1) It might be thought that the result is due to the precipitins and agglutinins contained in the immune serum or to bodies closely associated with these and simultaneously produced. (2) As the serum is not bactericidal, it might be assumed that it merely modifies the cocci in such a way as to make them susceptible to the natural defensive mechanism of the animal.

The difficulty with regard to (1) is that agglutinins, precipitins, &c., do not always run parallel with protective power. "For instance," to quote from the Rockefeller investigators, "we have had sera with high protective power and little or no "agglutinating power, and *vice versa*." So it seems necessary to recognise that there is some obscurity about the nature of these protective substances. Those who hold the "unitarian" view that all antibodies for a given type of pneumococcus are fundamentally one and the same would probably appeal to "antagonistic forces" or "buffer action" (*see* p. 58), the explanation being that the antibody which is present is both agglutinative and protective, but adverse physical conditions inhibit either the one action or the other. As for (2), it is only necessary to add that, in addition to direct action of the serum upon the cocci, there may be an indirect action, *i.e.*, the serum may stimulate the animal's resistance in some specific manner, as is thought to be the case in the therapeutic action of anti-anthrax serum.

The question then arises as to how much importance ought to be attached to these experiments on mice. There has been a natural temptation to hope that they will provide the key to the preparation of therapeutic sera, the idea being that the value of a serum will depend on demonstrable protective substances, and that therefore the desideratum is to produce sera of high titre in protection tests on mice. Unfortunately, the problem has been found to be much more complicated, apart from the obscurity, mentioned above, as to the real nature of the substances protective for mice.

First, there is a difficulty about active immunity. If the production of this condition always ran parallel with the serological development of the antibodies which protect mice, it might be thought that the mechanism of protection was the same in the two cases. But this parallelism does not always hold good. It may be found that the animal, though it has become immune against the homologous strain and against no other, has not developed any specific antibody which can be demonstrated, as shown by failure of tests for precipitation, agglutination, and protection of mice. Examples are also recorded where the converse result has been obtained; the animal undergoing immunisation has developed the usual specific antibody but has failed to become immune. It therefore appears that active immunity must be due to some factor other than these substances, and that such substances, when demonstrable, are concomitant effects rather than causes of active immunity,

though, by restricting dissemination of the cocci, or in other ways, they may be of subsidiary protective value.

At this point it may be argued that a sharp distinction should be drawn between active immunity, whether natural or acquired, and passive immunity, *i.e.*, the immunity conferred by administration of an immune serum. About the former, it may be conceded that unknown factors are concerned, but the problem of passive immunity may be regarded as less speculative, the presence of antibodies in the serum being all the explanation that is required.

There are certain difficulties about this view. The pneumococcus may, perhaps, be comparable to certain other parasitic bacteria against which therapeutic sera of undoubted value have been obtained. The mode of action of these sera is not settled; but it is at least clear that their value is not due either to precipitins and agglutinins or to such antibodies as are usually concomitants of these. A good example is to be found in the case of anthrax, where numerous investigators have shown that agglutinins and precipitins have nothing to do with the therapeutic value of the serum. Hence it cannot be accepted as a general principle that the antibodies which may be demonstrable in a serum suffice to explain passive immunity.

It is, however, possible that pneumococcal antisera are not, and never will be, comparable to anti-anthrax sera. It may be held that the work of the Americans on Type sera has reached the limit of what can be done in the case of pneumococci, that Type I serum, which appears to be effective in very large intravenous doses, acts in virtue of the known antibodies which it contains, and that the inefficacy of sera for Types II and III is due to the impossibility of producing the corresponding antibodies in sufficient concentration.

On the other hand, it may be thought that the production of better sera for other bacterial parasites still justifies the hope that the peculiarities of pneumococcal antigens and their corresponding antibodies may not prove an insuperable barrier to further progress.

What method is to be followed in the endeavour to discover some helpful factor? In addition to precipitins and agglutinins there are other well-accredited types of antibodies, each of which may be considered in turn, to see whether it yields a satisfactory explanation of active immunity against pneumococci. If the search proves unsuccessful, one may turn to other postulated types of antibodies (*e.g.*, anti-endotoxins or anti-aggressins), the existence of which is still a matter of controversy; something may be found in them which yields the desired clue; or an entirely new kind of antibody may be discovered.

In this search for a new biological factor, it is often assumed that the new antibody, when its existence is demonstrated, will be a special substance, probably of complex chemical and colloidal constitution, and that this "antibody complex" will differ

from other antibodies much in the same way as one antigen differs from another. This view, as I have suggested above (pp. 57–59) in discussing "colloidal balance," may turn out to be inadequate. And there are other reasons for not attaching exclusive importance to the protection afforded by specific antibodies.

In the case of an animal which is naturally immune against all varieties of pneumococci, how are the different antigenic variants disposed of? For reasons discussed above in relation to the "mosaic" theory (pp. 51–54), it does not seem likely that such an animal is provided with an indefinitely large supply of antibodies, each of which corresponds exactly with one or other of the antibodies produced artificially by immunisation with a variant. It seems more probable that the antigenic complex as a whole is attacked in some other way, and that some vulnerable point is found which has nothing to do with the particular structure characteristic of the variant.

In other species of animals, where natural resistance does not amount to absolute immunity, is spontaneous recovery from such a disease as lobar pneumonia due to the elaboration of specific antibodies against the variant causing the infection, or is it due to an independent mechanism, similar to that of the naturally immune animal though less rapidly effective? Probably the latter factor is of major importance, as it would be difficult to maintain that, at the time of the crisis, the development of specific antibodies against the particular variant is sufficiently pronounced to be regarded as the primary cause of recovery.

It must be admitted that the above arguments are not conclusive, because the future may lead to the discovery of some new kinds of antibodies which will provide the explanation required. But, in the meantime, it is desirable to call attention to some other aspects of the problem.

What is the explanation of the more important facts in the life history of pneumococci, such as the change from a saprophytic to a parasitic mode of life and the production of various forms of disease? Though at present the explanation is unknown, it at least seems clear that one cannot hope to arrive at it by simply ringing the changes on one idea, the antigenic stimulus of a foreign protein.

It is equally unlikely that the explanation of immunity towards pneumococci will ever be summed up as due to "pneumococcal antibodies," unless the term "antibody" receives some entirely new interpretation. Antibodies must be helped out by appealing to the aid of the mysterious forces known as "vital resistance." On pp. 62–63 I have discussed briefly attempts to simplify this mystery by identifying two of the forces with complement and phagocytosis. It is not likely that any strong claims will be made for "complement" as explanatory of resistance against pneumococci. But phagocytosis is often regarded as of high importance in the defences of the body against

these bacteria. This view is still popular, though there has been a good deal of dispute as to whether the phagocytes kill the pneumococci or devour them after they have been killed by some other means. Into this subject I have not attempted to enter in this report. As it will probably be a long time before any general agreement is reached on the importance of bacteriotropins and leucocytes in pneumococcal immunity, it would be unwise to await a decision on this point before considering other aspects of the question. Help might be derived from any differences or resemblances which can be demonstrated between acquired and natural immunity towards pneumococci.

Another question may be worth raising. If the more orthodox views about the significance of antibodies have to be modified, will any modification be needed in the current distinction between a vaccine, which is assumed to act as an antigen, and an immune serum, which is supposed to confer passive immunity by virtue of its antibodies ? With certain bacterial parasites it has been found that, in order to produce a therapeutic serum, the animal must be drenched with bacterial " antigen " long after the stage of active immunity has been reached. May not this treatment lead to the presence in the serum of some substance, perhaps a modified bacterial colloid, possessing the therapeutic property of a vaccine specially prepared so as to stimulate resistance promptly ? This may possibly be the case with the serum prepared by Preston Kyes (*see* pp. 14–16 of my preceding report).

Summary and Conclusions.

Method.

In reading literature on immunity it is found that most questions bristle with a perplexing variety of hypotheses, which cannot all be right. What is the best way of trying to get at the truth with the least possible delay ? There are at least four methods of recording scientific progress ; these are all useful and necessary, and they supplement each other. For convenience, I take them separately, though it is obvious that a writer may employ more than one.

(1) The investigator sets out his evidence in support of the hypothesis which he favours and then leaves his work to speak for itself, on the assumption that, in course of time, the weaker hypotheses will be neglected and the strongest will survive. In the case of a very brilliant piece of research, this method is successful. But, in the more common event of many rival contributions, no one of which is obviously pre-eminent, it takes too long to wait for the truth to speak for itself, on the principle of the survival of the fittest.

(2) The investigator not only pleads his own case, but endeavours to refute the arguments of his opponents. Scientific controversy, of course, is the most valuable way of clearing up the truth. The only trouble is that, as controver-

sialists are generally unwilling to give in, these disputes frequently end in a deadlock.

(3) The critic, assuming the judicial attitude of the unbiassed mind, sets out the evidence on the one hand and the evidence on the other hand; he generally concludes that more research is needed before the matter can be settled. This method, also, is excellent. It is acceptable and helpful to rival controversialists, because the critic presents each side fairly; and the critic has the satisfaction of feeling that he has not committed himself to any opinion which may ultimately turn out to be wrong.

(4) Hypotheses may be neither wholly right nor wholly wrong; the elements of truth which each contain may be pieced together and reconstructed. The critic who attempts reconstruction may be performing a useful task, but he is taking risks. The controversialists are not likely to be pleased, because nobody cares to be told that his theory is only good "in parts"; and if the critic's efforts at reconstruction happen to fail, the responsibility is entirely his own.

In dealing with the questions raised in this report, I think that method (4), though beset with pitfalls, ought not to be neglected.

General Principles.

In the endeavour to form a mental picture of chemical specificity as applied to bacterial antigens, I imagine that this specificity manifests itself in a variety of ways.

(1) To take the simplest case first, with some bacterial species there is a certain antigenic structure (A) which is common to each member of the species and is always demonstrable as the specific antigen.

(2) With other species, each member may possess a common nucleus (N) of antigenic structure, but to this nucleus there is always attached one or other of a variety of chemical groups, *a*, *b*, or *c*, &c.; and this attachment is of such a nature that antigenic properties are not manifested by N *per se* but only by the complex N*a* or N*b*, &c.; hence N*a* antigen reacts only with N*a* serum, not with N*b* serum.

(3) With other species, again, the conditions are more complex. There is not the uniformity of (1), due to the invariable dominance of A, nor the sharp diversity of (2), due to the dominance of different antigenic groups, *a*, *b*, &c. attached to a nucleus which is antigenically inert. Within the species there are different antigenic structures, *e.g.*, A, B or C, and to these may be attached a variety of chemical groups, *a*, *b*, *c*, &c.; but in this case the attachment is of such a nature as not to mask the antigenic value of the A, B or C. Hence, in comparing strains of such a species, if the term "species" be admissible for such an irregular group of organisms, resemblances will be found between any two possessing in common any one antigenic feature, A or *a*

B or *b*, &c.; for example, A*b* antigen will react with any serum containing either A or *b* antibody.

(4) There remains a fourth possibility. A group of organisms may come under category (3) in some respects but with the exception that, in certain instances, there is a reversion to condition (2); *e.g.*, the union of A with a chemical group *d* may mask the antigenic properties of A.

In discussing bacterial antigens it is often assumed that, if the structure of the bacterial cell were known in every chemical detail, this information would provide a complete record of antigenic properties, at least in their chemical aspect. I do not think that this assumption is correct. Antigen is a relative term, meaning capacity to produce antibodies in the living animal. When the bacterial protoplasm becomes modified by interactions taking place in the animal body, it seems to me probable that new antigenic capacities may emerge, and that these may depend upon peculiarities of chemical structure which were not represented in the molecules of the bacterial culture used for inoculation.

This view is in some respects incompatible with the "mosaic pattern" theory. I think this theory has been pushed a great deal too far as an attempted explanation of the nature of antibodies and their relations to antigens. Up to a certain point, the conception of multiple antigenic components is useful and is substantiated by experiment. It has been shown that some bacterial antigens are complex, and the fact that they consist of different antigenic components can be demonstrated by the selective action of the complex antibodies which they produce. So far the ground is safe. But, when one begins to represent this complexity in diagrammatic fashion by means of measured lengths and areas (showing that the antigen of a certain strain consists of $\frac{1}{2}a+\frac{1}{3}b+\frac{1}{6}c$, and so forth), one must beware of pitfalls, since it is very doubtful if such diagrams give any idea of the complicated chemico-physical reactions which really take place. And this is only the beginning of the danger. For a simple problem in serological classification three or four units may suffice to piece together all the different "mosaic" diagrams which it is desired to postulate; but complicated problems in immunity will require the introduction of a great many more units. If the diagrams are accepted in the former case, they ought to be accepted in the latter. And so one becomes confronted with mosaic patterns of ever increasing complexity. This, it appears to me, is the logical outcome of the theory. It leads to conclusions which I regard as unsubstantiated by fact and unsound in principle.

According to the biochemists, a good antigen should possess molecules which are not rapidly broken up in the animal body; otherwise, the antigen may disappear before it has persisted long enough to stimulate antibody production. I am not aware of any experiments showing that amongst bacterial antigens there are important differences in "staying power"; but I think this possibility is worth bearing in mind.

Proceeding from the chemical to the physical conditions which determine immunity reactions, it must be recognised that colloidal interactions in the living body are so complex that they are not amenable to experimental analysis. Sometimes this difficulty may be disregarded and it may suffice to assume, in general terms, that immunity reactions depend upon the combined influences of chemical affinity and physical adsorption. Thus, one may speak of the neutralisation of toxin by antitoxin, or the sensitisation of bacteria by agglutinins, precipitins or bacteriotropins, as simple facts due to the interaction of a definite antigen with a definite antibody, each of which is assumed to exist as a " pure " concrete substance. But, for the more difficult problems concerning susceptibility and resistance to disease, no simple explanation, such as the presence or absence of an appropriate antibody, is forthcoming. In such cases it is necessary to widen the field of enquiry, and here physical ideas about immunity come in for consideration, including the idea of a " balanced mechanism " which I have discussed at some length. Speculations of this sort are not conclusive; but they may be useful as correcting the tendency to frame all working hypotheses in terms of antigens and antibodies.

This tendency is often noticeable in discussions about immunity. Though it is customary to introduce prefatory remarks to the effect that very little is known about what takes place in the living body, and that further knowledge of natural immunity would throw fresh light on acquired immunity, the really business part of the discussion is frequently based on the maxim—find the right antibody to the right antigen, and then complement and phagocytosis will do the rest. But there are many facts about immunity which, though admittedly difficult to understand, at least indicate that this assumption needs revision.

Pneumococcal Antigens and Antibodies.

In applying the above general considerations to pneumococci, one first wonders if they help to explain why this species, though fairly well defined, contains such a large number of strains which are antigenically distinct from each other. Perhaps they are examples of condition (2) mentioned above (p. 49). If, in addition to serological differences, there was also strong evidence of serological relationship, the differences, or some of them, might be attributed to the " masking " of antigenic constituents exemplified in condition (4); but, since such serological interrelationship is not at all well marked, (2) seems preferable to (4) in application to the pneumococcal species. One is reluctant to think that each serologically different strain is really what might be termed a distinct serological species, such as a species in condition (1).

Can the pneumococcus be changed in the living body from one type into another ? It is theoretically possible that in acute disease there may be a tendency towards mutation into certain special types, and that in recovery there may be mutation into " atypical " varieties; but there is no satisfactory proof

that this is the case. On the other hand, the persistence of type in prolonged subculture does not show that mutation cannot occur in the living body.

The " mosaic " theory is of special interest because a highly elaborate explanation of pneumococcal antigens and antibodies has been based upon it. But there are many investigators who do not support this explanation, and it appears to me that the conception of a large and intricate mosaic pattern is not applicable to pneumococci and their antibodies. In fact, pneumococci seem less amenable to the " mosaic " idea than certain other bacterial species amongst which different strains or races show serological interrelationship.

Coming now to physical conditions in relation to immunity, these, no doubt, play an important part in the reactions between pneumococci and the living body; but they are involved in so much obscurity that it may appear questionable whether any profit can arise from discussing them. If satisfactory therapeutic sera for pneumococcal infections were available, and if their action could be shown to be due to demonstrable antibodies, discussion of these physical factors might be relegated to the more or less negligible class of " merely speculative " ideas. But these desirable results have not yet been attained, though further progress is to be hoped for. It may be possible, for example, to discover an antitoxic serum which will neutralise the toxic effects of pneumococcal invasion.

Meanwhile, in answer to the question which forms the subject of this report, I think the study of serological differences amongst pneumococci has not led to any final conclusions such as would justify the opinion that no serum will be therapeutically efficacious unless it contains an antibody corresponding to the antigen which is peculiar to the infecting strain.

Other sides of the question need consideration, into which factors of a physical as distinct from a purely chemical nature appear to enter. What is the significance of the bacterial capsule and of the mucinous material derived from disintegration of the capsules? A great deal has been written about " aggressins," " anti-aggressins," and " antiblastic immunity "; though these particular terms have dropped out of favour, the problems which they raise are of importance and are still unsettled. Then there are questions of cell permeability, as affecting capacities for bacterial invasion and as determining, on the part of the host, conditions which permit of the escape of antibacterial substances from leucocytes or other cells. Questions such as these, into which I have not entered in this report, cannot be settled by limiting the enquiry to a search for chemical affinities between antigens and antibodies.

My general conclusion, therefore, is that Neufeld's views about the significance of serological differences amongst pneumococci ought not to be accepted as final, though it must be admitted that the difficulties which these serological differences involve have not yet been overcome.

IV.—OBSERVATIONS ON THE DISTRIBUTION AND SEROLOGICAL CHARACTERS OF INFLUENZA BACILLI.

By W. M. Scott, M.D.

Scope of Enquiry.

The objects of the enquiry are—

(1) to determine the frequency with which influenza bacilli are to be found in the sputum or in the lung *post mortem* of patients suffering from respiratory inflammations (lobar pneumonia, catarrhal pneumonia bronchitis, influenza, &c.) and to compare this with their occurrence in the respiratory mucosa of normal persons;

(2) to investigate the serological reactions of the strains isolated from these various conditions; and

(3) to come to some conclusion from these data as to the pathogenic importance of the influenza bacillus. If it can be shown that particular morbid processes are associated with particular serological types of this bacillus, or that the spread of infection is associated with the prevalence in the respiratory passages of a particular type, a positive answer and useful epidemiological information will be furnished. If, on the other hand, it appears that the serological types of this organism are indefinite in character and incapable of correlation with disease, the result of the enquiry will be negative.

Material.

This may be divided into three sets :—

(1) Pathological specimens, chiefly from lobar pneumonia, investigated in conjunction with Dr. F. Griffith (*see* his report) and supplied during the period from April 1920 to the end of December 1921. The majority of the specimens were from patients treated in various Poor Law Infirmaries in London : my thanks are due, among others, especially to Drs. Baly and Perdrau of Lambeth, Dr. Harkness of Bermondsey, Dr. Brander of Hackney and Dr. Spurrell of Bow. Cases of influenzal pneumonia in South Wales, Sheffield and Bristol were responsible for a few specimens both in 1920 and 1921.

(2) Swabs from the nasopharynx of normal persons obtained during November 1918, November 1919 and November 1920. For these I am indebted to Mr. West, F.R.C.S., late Aural Surgeon to St. Bartholomew's Hospital, who was good enough to take swabs from the nasopharynx of out-patients attending the Ear and Throat Department, and to Dr. Baly, who allowed me to swab out-patients attending Lambeth Infirmary. By the kindness of the latter I was also able to swab patients suffering from influenza in the wards during the epidemic of November 1918.

(3) Specimens of sputum from acute cases of influenza in the wards of Bermondsey Infirmary during the epidemic of January 1922 and nasopharyngeal swabs from normal school children in Westminster during the same month.

Technique.

I have employed almost exclusively for the isolation and culture of influenza bacilli Fildes's pepsinised blood agar* which has proved much more satisfactory and convenient than any other.

On this medium the influenza bacillus outgrows most of the ordinary micro-organisms found in sputum. Its colonies appear after 24 hours incubation at 37° C. as sharply rounded flat discs of 1 to 4 m.m. in diameter, perfectly transparent and with no internal structure or iridescence. The only other bacteria which form colonies on this medium resembling these at all closely are the meningococci; the latter, however, are distinctly less transparent.

In addition to the production of these highly characteristic colonies, the features used for identification have been simple (1) the microscopical appearance of the bacilli as minute short Gram-negative rods and (2) the fact that no growth takes place on ordinary agar or other media free from blood pigment.

I have made no special study of the other cultural characters, but have noted that about two-thirds of my strains have produced abundant indol in broth containing pepsinised hæmoglobin,

* *British Journ. Experim. Pathol.*, I, p. 129. 1920.

while the remainder produced none. No other feature distinguished the indol-forming strains from those not forming indol and examples of both were found among groups serologically identical.

Meat water was found useful for the maintenance of the cultures, but, though some strains survived in it for over six months without subculture, others perished more rapidly and subculture was necessary about once a month.

For the preparation of agglutinating sera rabbits have been used exclusively; they were injected intravenously with living cultures of 24 hours incubation on Fildes's agar suspended in saline. The commencing dose should not exceed 5 milligrams of moist culture, but later much larger doses, up to 80 milligrams, may be given with safety. Satisfactory agglutination titres were usually obtained after six to eight weeks of immunisation; the sera were carbolised and stocked on reaching a minimum titre of 1 in 800.

Agglutination tests were performed in the water-bath at 50° C., readings being made after two hours at that temperature. The bacterial suspensions were made in phenol-saline, about 2 milligrams of moist culture being emulsified in each c.c. In a few instances, when the culture agglutinated spontaneously in normal salt solution, it was necessary to employ distilled water for suspensions. In all cases the test quantity was 0·5 c.c. of bacterial suspension mixed with the same volume of serum diluted with saline.

Frequency of Influenza Bacilli.

Table 1.

Isolation of Influenza Bacilli from Pneumonia, &c., April 1920 *to December* 1921.

Nature and Source of Material.	Total Specimens.	Positive.	Percentage Positive.
Sputum.—Lobar Pneumonia -	147	91	62
Lung P.M.—Lobar Pneumonia	14	6	43
Sputum.—Catarrhal and Influenzal Pneumonia -	29	21	73
Lung P.M.—Catarrhal and Influenzal Pneumonia -	7	7	100
Sputum.—Bronchitis - -	9	8	88

It is evident from Table 1 that influenza bacilli are of frequent occurrence in the tissues and discharges of pneumonia, whether of lobar character or catarrhal. As regards their abundance in the material only an approximate reckoning can be made. I have noted in the case of the 97 positive specimens from lobar pneumonia that 46 gave such abundant colonies that it might be said that the influenza bacillus was the predominating micro-

organism; 31 yielded a moderate number of colonies, while from 20 only one or two colonies could be identified. Of the 28 positive specimens from catarrhal and influenzal pneumonia 17, including 6 of those from the lung *post mortem*, furnished an abundant crop of typical colonies; 7 yielded a moderate number and 4 a few only. Of the 9 specimens from cases of simple bronchitis eight yielded influenza bacilli, five of them in abundance.

Table 2.

Isolation of Influenza Bacilli from the Normal Nasopharynx.

Population.	Date.	Total.	Positive.	Per-centage Positive.
Out-patients at St. Bartholomew's Hospital - - - - -	Nov. 1918	76	28	37
Out-patients at Lambeth Infirmary	Nov. 1918	63	27	42
" " "	Nov. 1919	34	11	32
" " "	Nov. 1920	13	9	70
Healthy school children in West-minster - - - - -	Jan. 1922	120	43	36

The figures preceding the last set represent individual out-patients, not suffering from respiratory disorder, from whose nasopharynx influenza bacilli were isolated from the only swab taken. It will be observed that during the periods of these examinations the influenza bacillus was quite a common inhabitant of the mucous membrane of the normal upper air-passages. In the case of the last group, the healthy school children, the swabs were taken from the nose and not from the nasopharynx and had already been used to inoculate Löffler's medium in the search for diphtheria carriers. The majority of the positive swabs, both from the children and the out-patients, gave cultures in which influenza bacilli were only moderate in number: swabs yielding abundant colonies were rare, as contrasted with cultures from sputum in disease.

Table 3.

Isolation of Influenza Bacilli from Cases of Influenza.

Source.	Date.	Total Cases.	Positive.	Per centage Positive.
Nasopharynx of Cases of Acute Influenza - - - - -	Nov. 1918	46	14	30
Sputum of Cases of Simple Influenza and Influenzal Pneumonia -	Jan. 1922	20	13	65

It will be observed, on comparing this Table 3 with Table 1, that the percentage of positive culture from the sputum of cases of influenza is not appreciably greater than from cases of lobar pneumonia and that the percentage of positive results from the nasopharyngeal swabs is within the range of positive results with swabs from normal persons; in the case of the influenza patients, however, the percentage of positive nasopharyngeal swabs is certainly too low: many were acutely ill, rendering the taking of the swab difficult and unsatisfactory.

Further, it is to be noted that the material for examination was obtained late in the disease in a considerable percentage of both groups. Among the 1918 cases 16 were examined within the first week of illness with four positives, 24 in the second week with six positives, and 6 in the third week with four positives. Among the 1922 cases 10 were in the first week of their disease with eight positives, and 10 had been ill from one to three weeks with five positives.

The data exhibited in these three tables indicate fairly clearly that the presence of the influenza bacillus in the sputum in inflammations of the respiratory apparatus may well be merely the consequence of its normal presence in the upper respiratory mucosa. It is true that its presence in the diseased lung *post mortem* might have more significance, but I have had no opportunity of arriving at an estimate of the frequency with which the bacillus may be found in the lung of persons dead from other causes: it is probable that its presence in the lung *post mortem* is not confined to cases of respiratory inflammation. Even its greater abundance in the expectorations of many of the cases of respiratory inflammation, as compared with the normal respiratory mucus, may be merely a consequence of the diseased condition and have no causal connection. It is evident that during epidemic and the shorter inter-epidemic periods influenza bacilli are so commonly found in the respiratory mucosa of the general population that no certain deduction in favour of their pathogenic activity can be drawn from their presence, even in abnormal numbers, in the discharges or local lesions of respiratory disease.

Association with Pneumococci.

The specimens from cases of pneumonia which were examined by me for influenza bacilli were also examined by Dr. F. Griffith for pneumococci, so that, by his courtesy, I may make a note on the association of these two micro-organisms in inflammations of the lung.

Of 161 specimens from lobar pneumonia, examined both for pneumococci and influenza bacilli, 84 yielded both, 64 yielded pneumococci only and 13 influenza bacilli only; making no allowance for accidental failures to isolate one or the other micro-organism, we still found both present in at least 52 per cent. of cases of lobar pneumonia. The great majority of the specimens were received during a period when influenza was not prevalent: in the earlier part of this period it was noted that, when " type "

pneumococci were present, influenza bacilli were often absent; but more recently this has not been so, as lobar pneumonias yielding " type " pneumococci have quite frequently yielded also abundant influenza bacilli. Nevertheless, of the 84 cases in which both bacteria were found, 32 gave " atypical " pneumococci, while of the 64 yielding pneumococci alone 12 only were " atypical " : the percentages are 38 per cent. as against 19 per cent.; *i.e.*, where the " atypical " pneumococcus was presumably the exciting cause, influenza bacilli were much more likely to be found than when the " type " pneumococci were concerned. The suggestion is that in some cases at least the association with influenza bacilli is necessary for the less virulent " atypical " pneumococci to produce lobar pneumonia. Pointing in the same direction is the fact that in broncho-pneumonia, which may be regarded as being in many cases a pneumococcal lesion of lower grade than lobar pneumonia, the pneumococcus found is almost (but not quite) invariably of the " atypical " group and is almost (but not quite) invariably associated with enormous numbers of influenza bacilli. Experimental difficulties have so far prevented me from attempting to demonstrate an enhancement of the virulence of " atypical " pneumococci for animals by association with influenza bacilli. I must therefore leave the question as still *sub judice*.

Serological Reactions.

Agglutination.

In Table 4 cross-agglutination reactions are exhibited between twelve strains of influenza bacilli and their respective sera. The figures represent in each case the dilution of serum producing complete deposit of the bacterial suspension, with the formation of clumps which remain visible to the naked eye on shaking up the deposit. In the instances where a ? is attached, the clumps were of a finer kind, barely perceptible to the naked eye on shaking. The titre for the homologous strain is in each case printed in heavy type (*see* page 82).

These cross-agglutination tests reveal certain relationships and also well-marked differences between the twelve strains. For example, serum Lister 3 agglutinates Fechger, L 45, S 1, Rosenhain and L 1A, though in no case at dilutions higher than 1 in 400. With the other strains this serum produces either no agglutination or fine agglutination disappearing on shaking.

The sera Self 1, L 10, L 36A and Fleming agglutinate well each other's homologues and also the strains L 45, Rosenhain and L 1A, but do not agglutinate Lister 3 or the other strains.

The serum B.S. 6 agglutinates almost exclusively its own strain and L 1A.

There are, thus, at least three antigenic components among these twelve strains, that predominant in Lister 3 which I have called " I," that common to Self 1, L 10, L 36A and Fleming which I have called " II," and that predominant in B.S. 6 and L 1A which I have called "" III.""

Table 4.

Agglutination Titres.

Source.	Serum.	Lister 3	Fechger.	L 45.	S 1.	Rosenhain.	Self 1.	L 10.	L 36A.	Fleming.	B.S. 6.	L 1A.	S 4.
Influenza Sputum	*Lister 3	**3,200**	100	400	100	400	0	0	0	?100	?100	100	0
Influenza Sputum	*Fechger	?100	**800**	0	0	?100	0	?100	0	?100	0	0	0
Lobar Pneumonia Lung - -	L 45	100	0	**1,600**	0	200	200	200	200	?100	0	?100	0
Influenzal Pneumonia Sputum	S 1	0	0	0	**800**	?100	0	0	0	0	?100	0	100
Influenza Sputum	*Rosenhain.	400	0	100	0	**800**	100	100	200	400	?100	400	0
Acute Cold Nasopharynx -	Self 1	0	0	100	0	100	**1,600**	800	800	200	0	?100	0
Catarrhal Pneumonia Lung -	L 10	0	?100	400	0	?100	800	**3,200**	800	200	0	100	0
Catarrhal Pneumonia Lung -	L 36A	0	0	400	0	100	800	800	**3,200**	200	0	100	0
Influenza - -	†Fleming	0	0	100	0	?100	400	400	200	**800**	200	100	0
Influenza - -	†B.S. 6	?100	0	0	100	?100	0	0	0	?100	**1,600**	800	0
Catarrhal Pneumonia Lung -	L 1A	200	?100	?100	100	400	0	200	0	100	1,600	**3,200**	100
Influenzal Pneumonia Lung -	S 4	0	0	0	100	0	0	0	0	0	0	0	**800**

* I am indebted to the Lister Institute for these three strains.
† For these two strains I wish to thank the Royal Army Medical College.

As shown by its negative agglutination reactions with the other sera the strain Lister 3 is almost free from the II and III components: the strains Self 1, L 10, and L 36A are pure II, and the strain B.S. 6 contains only traces of I and II antigens.

But the strains Fechger and S 1, while containing the I antigen in greater amount than II or III, yet do not contain it as the predominating component. Fechger produces a serum agglutinating none of the other eleven strains to a significant degree (it picks out a few from the larger collection of strains from pneumonia, &c.). Its main antigen is evidently different from I, II, and III.

Rosenhain and L 45 react with both Lister 3 and the II sera, *i.e.*, they contain both I and II antigens in significant amounts.

S 1 reacts with the sera of both Lister 3 and B.S. 6, *i.e.*, it contains a certain amount of both I and III components, but its serum agglutinates the other eleven strains hardly at all. Like Fechger, it evidently possesses another quite different antigen as the predominating constituent, and its serum similarly fails to agglutinate decisively the other strains except S 4: it agglutinates well, however, a few strains isolated from other cases of catarrhal pneumonia and, as will be seen, may represent an epidemic type.

Fleming, though its II component is strongly marked, has affinities with B.S. 6 and L 1A, *i.e.*, it contains also some of the III antigen.

L 1A, though the III component predominates in it, has quite significant amounts of both I and II and, accordingly, its serum, though a strong III agglutinator, definitely agglutinates both Lister 3 and L 10.

S 4 does not agglutinate strongly with any but its own serum and its serum fails to agglutinate any of the other strains except S 1. Its affinity to S 1 is further indicated by the fact that S 3, a strain isolated from a case of influenzal pneumonia in the same locality, is agglutinated by both S 1 and S 4 sera and by these alone.

If one assumes that these twelve strains are at all representative of the influenza bacillus as a species, it is evident that, though it is possible to differentiate certain antigenic components, yet the species has as its most pronounced serological feature a great diversity in antigenic character.

Absorption of Agglutinin.

The three antigens indicated by the cross-agglutination tests as being fairly widely distributed in the species are readily demonstrable by the test for absorption of agglutinin. In fact, in the majority of cases in which agglutination occurs with the formation of permanent clumps visible to the naked eye, it is possible to show that contact with the agglutinable bacillus removes agglutinin from the serum, *i.e.*, that an antigen is present in the agglutinated strain similar to one present in the strain with

which the agglutinating serum was produced. With certain agglutinable strains, however, the amount of agglutinin removed is insufficient to lower appreciably the titre of the serum for the homologous strain : in such cases it is usually possible to show that the titre for another related strain is partially or completely abolished. The Table 5 illustrates the effects of contact with various agglutinable strains on the agglutinating power of the serum.

Table 5.—Absorption of Agglutinin.

4 mg. moist culture mixed with each 1 c.c. of 1/50 dilution of Serum (V. B.S. 6).

	B.S. 6	Lister 3	L 1A	L 18	L 36	L 39	S 1
Titre before absorption	*800	100	400	400	100	100	200
After absorption with B.S. 6	0	0	100	0	0	0	0
After absorption with Lister 3	?800	0	200	200	100	100	?100
After absorption with L 1A	200	0	0	100	0	0	100
After absorption with L 18	?800	0	100	100	?100	?100	100
After absorption with L 36	400	?0	0	100	0	0	100
After absorption with L 39	?800	100	400	200	100	0	100
After absorption with S 1	800	0	100	200	200	100	0

* A sample of serum taken before the final titre was attained.

It is evident from Table 5 that the strains L 1A and L 36* contain considerable quantities of the same antigen as is contained in B.S. 6, the strain with which the serum was produced.

In addition, it may be noted that, though Lister 3 removes only a trace of the B.S. 6 agglutinin (agglutination at 1 in 800 barely complete), it reduces by half the titres for L 1A and L 18, showing that part of the antigenic complex responsible for the agglutination of these two strains is present in Lister 3 ; further, absorption with S 1 does not reduce the agglutinin for the homologous strain B.S. 6 but removes all the agglutinin for Lister 3 and a considerable part of that for L 1A and L 18, *i.e.*, the strains S 1, Lister 3, L 1A, and L 18 have certain antigenic components in common.

Speaking generally, one may say that the relationships between strains indicated by agglutination reactions are confirmed by their powers of removing agglutinin, and that, therefore, these relationships depend on similarities in antigenic structure.

* (L 36 is a strain isolated from the same case of lobar pneumonia as L 36A : the former came from the sputum, the latter from the necrotic lung *post mortem* about three weeks later. The former is a III strain, the latter one of the typical II strains.)

DISTRIBUTION OF THE COMMONER ANTIGENIC COMPONENTS.

Complete serological classification of my strains of B. influenzæ has proved to be impracticable owing to the multiplicity of their antigenic components. I have classified them, therefore, into groups according as one or other of the commoner antigens revealed in Table 4 is present in a strain in significant amount. In the case of many strains more than one of the three common antigens can be detected: such strains I have classified as "mixed." There remains a large residuum in which these antigens are absent or present in insignificant amount: these strains I have classified as "individual."

The presence of the group antigen has been taken as established when agglutination of the strain with the production of permanent clumps, visible to the naked eye, has occurred in the group serum diluted 1 in 100. A sufficient number of strains (20) have been tested for absorption of agglutinin to make this assumption reasonable. The group sera employed have been—for group I serum Lister 3, for group II serum L 10, for group III serum B.S. 6.

The majority of the strains classified have been tested also with the other nine sera of Table 4: the results have confirmed the grouping: in a few cases close serological relationship has been demonstrated with strains such as Fechger in which the predominant antigen is one of relatively rare occurrence; such strains have, of course, remained in the "individual" group.

It must be clearly understood that the classification shown in Tables 6, 7 and 8 is not a classification into types such as exist in the case of the pneumococcus, nor even into such as have been found in the meningococcus. It indicates only the extent of the distribution of certain antigens in the species as found at the present time. In many of the strains which have been grouped under the headings I, II, III and "Mixed" the dominant antigen has probably not been detected, being a complex peculiar to the strain itself: that is to say, if sera were prepared with such strains they would agglutinate the homologous bacilli to a much higher titre than any of the strains selected as representative of the group. Strains of this kind differ only from those grouped as "individual" in possessing a certain amount of one or more of the three antigenic components which have been found to be of more general occurrence.

Table 6.

Distributions of the Commoner Antigens among Strains isolated from Sputum and Lung from April, 1920, *to December*, 1921.

I	II	III	Mixed.	Individual.	Clinical Condition.
10	18	8	28	33 [=34 per cent.]	Lobar Pneumonia.
7	7	5	1	8 [=28 per cent.]	Catarrhal and Influenzal Pneumonia.
4	2	—	2	—	Bronchitis.

Distribution in Pneumonia, &c.

Table 6 shows the extent to which the three antigens selected for grouping occur among the strains isolated from the common inflammations of the respiratory apparatus. In the pneumonias about a third of all the strains contain either none or only insignificant amounts of these antigens. The few strains from simple bronchitis on the other hand all fall into groups.

Table 7.

Distribution of the commoner Antigens among Strains isolated from the normal Nasopharynx.

I	II	III	Mixed.	Individual.	Population.
7	6	3	9	35 [=58 per cent.]	Out-patients at St. Bartholomew's and Lambeth, &c., in 1918, 1919 and 1920.
5	—	—	1	—	School Boy Contacts of Influenza in 1920.
18	6	—	1	18 [=42 per cent.]	School Children in Westminster in January, 1922.

Distribution in the normal Nasopharynx.

The first series of 60 strains includes 45 of those already referred to (*vide* Table 2) as having been obtained from out-patients at St. Bartholomew's Hospital and Lambeth Infirmary in 1918, 1919 and 1920, the remainder being miscellaneous from staff, etc. The small series of contacts represent the 6 strains isolated from 25 boys at a Public School in which a mild outbreak of influenza was in progress, while the series from school children in Westminster is comparable with the latter series in that the swabs were taken at the height of the epidemic of January, 1922.

The first series is interesting as showing a high proportion of "individual" strains, 58 per cent. as compared with 32 per cent. among strains from lobar pneumonia and still lower percentages among the other categories. The point of interest in the other two sets is the high proportion of the group I strains in both. Here there is certainly some indication that a particular antigen may be characteristic of epidemic strains of the influenza bacillus.

Table 8.

Distribution of the commoner Antigens among Strains isolated from Cases of Influenza.

I	II	III	Mixed.	Individual.	Source.
1	5	2	4	2 (out of 14)	Nasopharynx of Influenza cases in November, 1918.
3	3	2	—	5 (out of 13)	Sputum of Influenza cases in January, 1922.

Distribution in Cases of Influenza.

Both the series are unfortunately very small, so that conclusions are difficult to draw. It will be observed, however that, while in the 1918 epidemic a high proportion of the strains contained the group antigens, in the 1922 epidemic the proportion was lower, being about the same as in the strains isolated from pneumonia during the inter-epidemic period; *i.e.*, there is no evidence of a special pathogenic type.

The Question of Epidemic Types.

Do epidemic types occur ? In this connection certain observations, though not conclusive, are perhaps worth recording. The strains S1 and S4 (*vide* Table 4), as well as two other strains S2 and S3 with which I have not prepared sera, all came from cases of influenzal pneumonia during an outbreak of influenza in Sheffield in May 1920. As has been remarked, these S strains, though exhibiting, at least in the case of S1, some slight relationship with the other strains used as standards, were conspicuously "individual"; their predominant antigen did not occur among the other strains nor has it appeared among the strains isolated in the London area from ordinary non-influenzal pneumonias during the inter-epidemic period 1920 to 1921.

In April 1921, however, Dr. Nixon of Bristol sent me a specimen of lung from an infant of 1 year who died of catarrhal pneumonia: the consolidation was typically lobular. An abundant growth of influenza bacilli was obtained which agglutinated strongly with S1 serum and absorbed the agglutinin of this serum completely (a few pneumococci of the "atypical" group were also found). There was no epidemic prevalence of influenza in Bristol at the time, but ten days later a specimen of sputum was received from Bristol from a case described by Dr. Nixon as typical "violet" influenzal pneumonia with great prostration. This again yielded an abundant growth of influenza bacilli (but no pneumococci) agglutinating strongly with S1 serum and absorbing a high proportion of its agglutinin. No connection could be established between these cases, and no further specimen from influenzal pneumonia was received from Bristol till July 1921, when again sputum from a case of "violet" pneumonia yielded an abundant culture of influenza bacilli agglutinating with S1 serum and with a serum prepared with the strain from the second Bristol case. At the same time, however, a similar strain was isolated from a case of ordinary lobar pneumonia in Birmingham, indicating at once that the S1 antigen was not confined to strains from influenzal pneumonia. Furthermore, during the epidemic of January, 1922, the S1 antigen has appeared in strains from two healthy school children and from one case of ordinary lobar pneumonia in London, while among the strains from cases of influenzal pneumonia during this London

epidemic the S1 antigen has been conspicuously absent. Hence, its association with the Sheffield and Bristol cases, which appeared so interesting, may be merely accidental, and I am not inclined now to lay stress on this serological characteristic as a possible indicator of special pathogenicity.

Discussion.

The question may now be discussed whether the results which I have recorded throw any light on the much debated question whether Pfeiffer's bacillus is the cause of influenza.

I have already remarked that the presence of influenza bacilli, even in large numbers, in the discharges or local lesions of respiratory disease is not a sufficient argument on which to establish their primary pathogenic activity, since this correlation may represent a secondary invasion, the consequence of their prevalence in the normal respiratory mucosa.

In the case of the meningococcus there is a similar wide distribution in the nasopharynx of the general population, which has been particularly noted during epidemic prevalence of cerebro-spinal fever, while the pneumococcus is at all times a common inhabitant of the upper respiratory tract.

Yet the pathogenicity of these two micro-organisms is not seriously questioned because, (1) in both cases they are present in disease, accompanied by characteristic lesions, in tissues from which they are normally absent, and (2) in both cases recent investigation has shown that the majority of the strains found in disease fall into a few well-defined serological groups.

In the case of the influenza bacillus the first condition has not been established and, so far as my investigation goes, there is no evidence of the second being fulfilled; the serological characters of the strains isolated from cases of influenza are almost as diverse as those isolated from other sources, including normal persons.

There is thus no support afforded by the serological reactions in favour of a primary pathogenic relation existing between Pfeiffer's bacillus and influenza. Can it be said on the other hand that the great diversity in serological type excludes the influenza bacillus from being a primary agent in infection? Again, I think, the answer must be in the negative, at least until the meaning of the similar diversity among other bacterial species which are certainly concerned in the production of epidemic diseases, has been satisfactorily explained. It is conceivable that this very diversity in the case of the B. influenzæ species may indicate an active evolution, aiming finally at the emergence of fixed pathogenic types such as are from time to time evolved among the meningococci. Denial of epidemiological importance to the influenza bacillus appears to me, therefore, as premature as the assertion of its exclusive relation to the production of epidemic influenza.

Conclusions.

(1) During the four years 1918-1921 influenza bacilli have been found in the normal nasopharynx in over 30 per cent. of the persons I have examined.

(2) During 1920-1921 influenza bacilli have been found in the sputum or lung in about 60 per cent. of cases of lobar pneumonia, in 88 per cent. of cases of simple broncho-pneumonia and bronchitis and, during the epidemic of January 1922, in 65 per cent. of cases of influenza and influenzal pneumonia.

(3) Agglutination tests with specific monovalent rabbit sera have revealed great diversity in serological type. An attempt at partial classification has shown that certain antigenic characters occur in a proportion of the strains from all these different sources.

(4) There is no satisfactory evidence of special serological types being associated with disease nor with epidemic prevalence of influenza.

(5) The serological diversity of influenza bacilli cannot be used as an argument either in favour of or against their etiological relationship to influenza.

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